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DEPARTMENT OF PATHOLOGY

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DEMONSTRATION OF SPECIMENS RELATING TO THE TRANSMISSION OF ARTIFICIAL CULTURES OF LEISHMANIA INFANTUM TO MICE AND RATS,

In the Section of Tropical Medicine at the Annual Meeting of the British Medical Association, Liverpool, 1912.

By H. BAYON,
Beit Memorial Research Fellow, Lister Institute of Preventive Medicine.

NOVY, in 1908, was the first to publish the successful transmission of artificially cultivated *Leishmania infantum* to animals. By repeatedly injecting a dog during the course of five months with the contents of 270 culture tubes of Nicolle's strain of *Leishmania infantum*, he succeeded in finding, eighteen days after the last injection, a large number of protozoa in the spleen, liver, and bone marrow. The parasites were regained in pure culture.

A year later, in conjunction with McJunkin and Schule, the same author announced having infected five dogs, to one of which he had injected at one time the contents of twenty tubes.

Franchini injected 1 c.cm. of a culture of *Leishmania infantum* intraperitoneally into a guinea-pig, and got an infection of the animal apparently without further trouble.

Delanoe, and later Visentini, have, however, found that *Leishmania infantum* cultures injected intraperitoneally into mice and guinea-pigs are very soon destroyed, and Visentini was able to show that in the peritoneum of the guinea-pig, after an hour and a quarter, it was not possible to regain the protozoa by culture on blood-agar.

That dogs and monkeys are receptive for the "virus" (in contrast to the culture) of infantile kala-azar is a well-known fact. This experiment has been so often repeated by different workers after Nicolle's, Comte's, and Manceaux's first experiments in 1908 that it may be considered an established fact.

On the other hand, cats, guinea-pigs, and rats have been shown by Nicolle and others to resist even the injection of virus direct from the patient, so that Franchini's extremely prompt result appears to await independent confirmation.

With the intention of getting a strain of *L. infantum* virulent for animals I carried out some experiments, the corresponding slides of which I am now demonstrating.

The strains of *L. infantum* I made use of were kindly given me by Dr. Visentini, who at that time was working at the Lister Institute. One strain originally came from Tunis, having been isolated there by Nicolle; the other culture had been made in the first place by Professor Jemma of Palermo.

Both cultures grew abundantly and easily on rabbit-blood agar within five days from subculture. After fifteen to twenty days numerous rounded, non-flagellate forms are present, also numerous disintegrating, that is vacuolated and badly-staining flagellate shapes are to be seen. I have succeeded in getting subcultures also from such old tubes.

An initial series of experiments with other protozoa was carried out with the intention of showing, by microscopical examination combined with animal injection and artificial culture, to what extent the natural immunity of an animal affected the virus injected; also with the further purpose of demonstrating similar or analogous results with the cultures of *L. infantum*, should the animals injected show themselves highly immune or resistant, as was to be expected from Delanoe's and Visentini's experiments.

I was therefore comparing an immunity I knew to be definite with one whose degree I still was doubtful about.

For this purpose blood from rats infected with *T. rhodesiense*, a protozoon which we have every reason to believe is extremely difficult to transmit to birds, was injected intramuscularly into five sparrows. Their heart's blood and ground-up organs were reinjected into rats after intervals varying from twenty-four hours to five days.

In no case did the rats show any infection, so that I could conclude from these experiments that naturally immune animals were capable of completely destroying certain protozoa in their circulation within twenty-four hours.

The possibilities of the direct cultural method were now tried. It was not possible to use *T. rhodesiense* in this case, as its culture is still a matter of great uncertainty, so an easily cultivable protozoon—namely, *T. lewisi*—was experimented with. To avoid confusion with bird trypanosomes, mice were used as experimental animals, also in consideration of the fact that their immunity against *T. lewisi* is not very marked, and therefore the behaviour of protozoa towards natural immunity of an inconstant type could be observed.

Three mice were injected intraperitoneally with 1 c.cm. of rat's blood, showing very numerous *T. lewisi.* The rat had been infected with the blood taken from a wild rat. Cultures were taken from the heart's blood, peritoneum, and organs of the mice. Microscopical preparations were also made. The mice were killed after eight hours; one showed a few trypanosomes of the *lewisi* types in the blood of the heart. Partially disintegrated trypanosomes were found in the peritoneal fluid of all three, together with many motile and apparently faultlessly staining parasites. All culture tubes remained sterile.

The limitations of the cultural method appeared evident in this case, where a temporary infection was apparently in course. As a matter of fact, Rondsky has shown that mice can be infected with *T. lewisi* under certain circumstances.

I now proceeded to attempt to infect mice and rats with *L. infantum*, bearing in mind that, according to the analogy of my previous experiments, should the animals be absolutely immune against this organism, I could not expect with probability a culture even after such a short time as eight hours, and certainly not after twenty hours, as the extremely delicate method of rat inoculation had failed with *T. rhodesiense*, a protozoon which infects rats in minimal quantities, and even after forty-eight hours after the death of the host (Blacklock).

Three mice were injected intraperitoneally with 1 c.cm. each of six to ten days old cultures. The animals were killed four hours after injection; smears were taken from the peritoneal fluid and organs, and cultures from the blood, organs, and peritoneal fluid. A culture tube taken from the peritoneal fluid was successful, showing numerous *Leishmaniae* a fortnight after the others remained sterile.

In the smears taken from the peritoneal fluid the following appearances were evident:

The flagellate parasites were taken up by big mononuclear cells and disintegrated in the fashion described by Delanoe and Visentini. A certain number were phagocyted by neutrophile, polymorphonuclear leucocytes and presented the identical appearance of the rounded or oval non-flagellate stage, with or without the short rhizoplast rod which is met with in the organs of persons affected with kala-azar. I have placed a specimen showing this condition under the microscope.

Various stages of disintegration are to be seen in these specimens, and I can confirm Visentini's observation of the resistance of the flagellum to disintegration and the rounding-up of the protozoa in the protoplasma of the mononuclear cells. The observation I intend emphasizing to-day is that the rounded or oval stage of *L. infantum* which is seen in cultures resists disintegration much longer and more effectually than the flagellate stage.

This rounded shape is not in all cases a result of the transformation of the leptomonad phase, because the leptomonas condition, once enclosed in the plasma of the leucocyte, gets soon digested and loses its staining properties, whilst these rounded bodies stain sharply and clearly.

Does this observation agree with any of the facts hitherto known? Certainly it does, for we know that the "virus" from a patient affected with *L. infantum* infects animals, or in any case is capable of transmitting the disease without exceptional difficulty to dogs and monkeys, whilst the cultures are only able to infect animals after repeated injections extended over a relatively long period of time. The "virus" consists, however, of rounded, oval, non-flagellate phases of the parasite, whilst in cultures the leptomonad or flagellate stage is by far the most predominant one.

If we turn, then, to the question of insect vectors, we find that the only experiments that have any satisfactory results to show are those of Basile, with the transmission by means of *Pulex serraticeps* and *Pulex irritans*. On examining Basile's specimens, which this author kindly showed me in Rome, and one of which he has sent me for demonstration purposes, we find, again, that in the naturally infected *Pulex serraticeps* from the dog the rounded, non-flagellate stage predominates.

Similar observations are recorded by Alvarez and De Silva, who found in *Pulex serraticeps* taken off a dog naturally infected with leishmaniasis, round and flagellated stages of the protozoon. The faeces of these fleas showed the round phase only, an observation not without interest in view of the recent work of Brumpt, who has found that the faeces of *Conorhinus megistus* are capable of infecting guinea-pigs with *Schizotrypanum cruzi*.

Alvarez and De Silva did not find any protozoa in the fleas taken from healthy dogs.

It is, of course, quite easy to see that none of these observations have a decisive value, but still they seem to point to the fact that the rounded or oval stage of *L. infantum* is the infective phase of the protozoon, and that in any case the immunity of mice against the cultural stages that imitate this rounded stage is not very great, otherwise culture would not have succeeded four hours after injection into the peritoneum of the animal.

Regarding the experiments of Franchini with *Anopheles maculipennis*, we find that also here a non-flagellate stage appears to be considered infective. However, in these experiments no positive results in animals were performed; not only that, but the drawing and specimens I have seen show the parasites clearly disintegrating in the gut of the mosquito. In the last paper published in the *Annals of Tropical Medicine and Parasitology*, 1912, vi, pp. 41–52, we find that in no case is mention made of the time after infection or feeding when the mosquitos were examined. This is an important point —the principal one, in fact.

Referring to the plates accompanying the paper, we find that *de facto*, incorrect or incomplete in detail as the pictures may be, still they show clearly that the parasites are disintegrating—an opinion that is shared by Martin Meyer on page 441 of his chapter on "Leishmanien" in the seventh volume of Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen*.

This may appear severe criticism, but I can but refer to drawings appearing on Plates III and IV, on which in no case, except one, the flagellum is represented penetrating the protozoon, or rising from a basal granule near the kinetonucleus, as it does in reality, but instead, where present at all, it is seen to fade away feebly in the periplast of the flagellates—an appearance that can only be due to bad staining, incomplete observation, or both combined.

Of the many "dividing forms" figured in the plates, No. 14 on Plate III, and Nos. 7, 14, 17, 21 on Plate IV can scarcely with fairness be called "dividing forms." On this matter I can safely await the judgement of any one versed in microscopy.

I have been obliged to pay so much attention to Franchini's paper because its results, and the results of his school in general, are confusing the issues by a series of observations which do not agree with those made by other workers in the field.

The experiments with the intention of getting a strain of *Leishmania infantum* pathogenic for mice from cultures are being continued, for so far I must admit not having succeeded in finding any *Leishmaniae* in rats and mice which were examined twenty days after repeated injections of cultures.

For the moment I should like to venture the suggestion that the rounded or oval, non-flagellate stage of the *Leishmania* protozoon is the infective phase, and that the significance of the leptomonad forms in cultures is, that they correspond to the developmental, non-infective, multiplicative phases in the invertebrate host.

Somewhat analogous conditions seem to hold good with Chaga's *Schizotrypanum cruzi*, where, however, a flagellate, trypaniform phase, in the "virus" is infective, but cultural forms non-infective.

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THE FACTORS CONDITIONING THE VELOCITY OF DISINFECTION

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The foundations of study of disinfection were laid by the work of Pasteur and Lister when these masters demonstrated that the influence of certain preservatives lay in preventing growth of putrefactive bacteria on the one hand and of disease organisms on the other. Their work was subsequently amplified by many investigations on similar lines. A great advance was, however, made by Robert Koch (1886), who was the first to institute systematic experiments with pure cultures of bacteria; by means of his "thread" method, using anthrax spores as test organism, he made a comparative study of all the known disinfectants, among which he devoted special study to the remarkable disinfectant properties of the salts of mercury.

The first occasion on which the principles and accurate quantitative methods of the chemist were applied to the study of disinfection was in 1897, when Krönig and Paul published their classic researches on disinfectants and disinfection thereby raising the subject to a higher plane of scientific enquiry than it had previously occupied. For example, they first called attention to the connection between disinfectant action and electrolytic dissociation in the case of many metallic salts, alkalies and acids.[1] They also estimated relative germicidal power by a comparison of disinfection-rate in the various cases, and by means of their garnet method, using anthrax spores as test organism, they were able to form an estimate of the initial number of bacteria present and of the survivors from time to time when exposed to the action of various germicides. They thus showed that disinfection was a gradual process, depending for any particular germicide upon the temperature and the concentration of the latter, but they made no further use of their results in formulating a theory of disinfection.

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[1] The work in this direction was later made complete by the researches of Winslow and Lockridge (1906).

On further examination, however, of the valuable series of data given by Krönig and Paul, disinfection is discovered to be an orderly time process for if numbers of surviving bacteria as ordinates are plotted against time as abscissae, the experimental points lie upon fairly smoothed curves.

The form of these curves suggested the existence of a logarithmic relation between the two variables, time on the one hand, and the number of surviving bacteria on the other. In fact the curves appeared to be very similar in form to that expressing the course of a so-called "uni-molecular reaction" and the equation

$$-\frac{dC}{dt} = KC,$$

$$\text{or } \frac{1}{t_2 - t_1} \log \frac{C_1}{C_2} = K, \text{ a constant}$$

was found to be applicable to the case of disinfection, if, in place of the terms C_1 and C_2 expressing concentration of reacting substance, numbers of surviving bacteria were inserted, thus

$$\frac{1}{t_2 - t_1} \log \frac{\overset{N}{\cancel{p_1}}}{\underset{N}{\cancel{p_2}}} = K$$

Where n_1 and n_2 are the numbers of bacteria surviving after times t_1 and t_2 respectively.

Madsen and Nyman (1907) working simultaneously with myself but independently, also discovered the existence of the above relationship from Krönig and Paul's results and, using the method of the latter, verified the law for the destruction of anthrax spores by hot water.

In the course of my own work (1908 and 1910), I was able to confirm the logarithmic nature of the disinfection process for vegetative organisms as well as spores, using a variety of germicides, including heat. In the present paper I propose to give the results of a few typical experiments as examples and then to proceed to a discussion of the probable explanation of the above relationship.

EXPERIMENTAL

The "garnet" method of Krönig and Paul, in which the organisms are presented to the disinfectant as a film dried upon the surface of a garnet, is unsuitable where vegetative bacteria are concerned. In my own experiments, therefore, this method was abandoned in favour of a modification of the "drop" method of Rideal and Walker (1903). A suitable small measured quantity of a diluted broth culture (24 hours at 37°C) of the organism under examination (or of a heated emulsion from an old agar culture in case of anthrax spores) was added to a measured quantity of disinfectant solution, placed in a thermostat at the required temperature. At definite intervals of time measured samples were withdrawn by means of special capillary pipettes fixed into the mouth of the disinfection tubes and immediately plated on gelatine or agar. The numbers of colonies were counted after incubation for 4–5 days at 20° C, or 2 days at 37°C respectively.

The exact details of the method employed, and the modifications in case of disinfection by means of hot water, as well as of the expedients adopted to secure constancy of resistance in the test organisms will be found in the references given above.

Table I*a* gives the results of an experiment with anthrax spores and phenol at 33.3°C. In the case of this material (which had been previously freed from vegetative forms by heating to 80°C for ten minutes) disinfection proceeded with almost mathematical precision as a "reaction of the first order," Table I*b* contains the results of an experiment of Krönig and Paul with anthrax spores and mercuric chloride for comparison. In the last column of each Table are given the values of the velocity constant, calculated on the assumption that disinfection-rate is always proportional to the concentration of surviving bacteria; the values obtained show very fair constancy.

TABLE I *a.*

Disinfection of Anthrax Spores with 5% phenol at 33.3°C.

(See H. C. (1908), Table II, p. 99.)

Time minutes $=t$	Mean number of bacteria surviving in one drop of disinfection mixture, $=N$	$\text{Log}_{10}N$	*$K = \frac{1}{t_n - t_0} \frac{\log N_0}{\log N_n}$
$0 = t_0$	$439 = N_0$	2.642	—
30	275.5	2.439	.0068
75	137.5	2.138	.0067
120	46	1.663	.0082
180	15.8	1.199	.0080
246	5.45	.736	.0077
300	3.6	.556	.0069
420	0.5	−.301	.0070

---

* In this and all subsequent tables, values of K from the expression $\frac{1}{t_n - t_0} \log \frac{N_0}{N_n}$ are calculated with Briggs' logarithms in place of natural logarithms.

TABLE I *b*.

Disinfection of Anthrax Spores with 0.11% *mercuric chloride at* 18°C.

(See Krönig and Paul (1897), Table IX, p. 26.)

Time, minutes $=t$	Mean number of bacteria surviving $=N$	$\mathrm{Log}_{10}N$	$K=\frac{1}{t_n-t_0}\log\frac{N_0}{N_n}$
$10=t_0$	$2027=N_0$	3.307	—
15	672	2.827	.096
20	564	2.751	.056
25	382	2.582	.048
30	251	2.400	.045
35	179	2.253	.042
40	138	2.140	.039
45	101	2.004	.037
50	80	1.903	.035
60	39	1.591	.034
70	6	.778	.042
80	3	.477	.040

This logarithmic relationship can also be expressed graphically; if the logarithms of concentrations of surviving bacteria as ordinates be plotted against time as abscissae, the experimental points will be found to be upon straight lines. (See Figures 1 and 1b on page 172.)

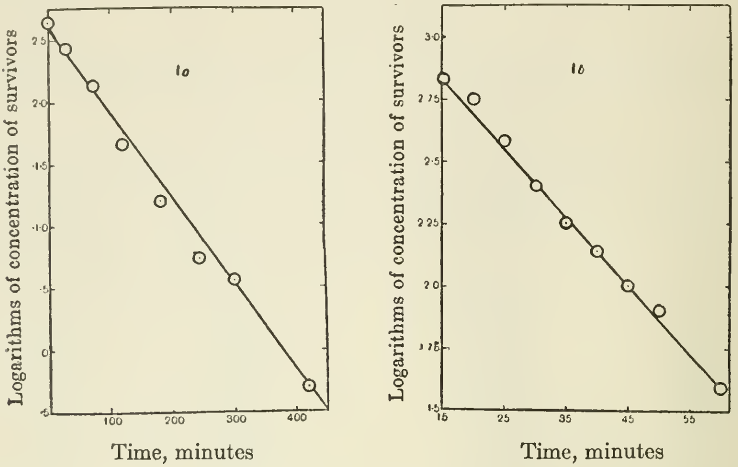

Fig. 1. (*a*) Disinfection of anthrax spores with 5% phenol at 33.3°C. (See Table 1a above).

(*b*) Disinfection of anthrax spores with 0.11% mercuric chloride at 18°C. (See Table 1b above).

Similar results were obtained for disinfection with Phenol of *B. typhosus* and *B. coli commune:* the results of a typical experiment with the former organism are shown in Table II and fig. 2 on page 173.

TABLE II.

(H. C. 1910 Table II)

Disinfection of B. typhosus with 0.6% *phenol at* 20°C.

Exp. 8. 1. '10.

Time, minutes $=t$	Amount of sample taken, drops	Numbers on plates (gelatine)	Mean	Mean no. of bacteria present in 1 drop disinfection mixture, $=N$	$\text{Log}_{10}N$	$K = \frac{1}{t_n - t_0} \log \frac{N_0}{N_n}$
$0.5 = t_0$	1	236 218 240	231.3	$231.3 = N_0$	2.364	—
1.5	1	188 215 167	190	190	2.279	.085
2.6	1	160 157 184	167	167	2.223	.067
4.6	2	215 242	228.5	114.2	2.058	.075
6.6	2	139 172 156	155.7	77.8	1.891	.077
10.5	3	100 93 91	94.7	31.6	1.500	.086
15	3	33 26 43	34	11.3	1.053	.090
20	5	14 14 8	12	2.4	.380	.102
30	10	4 3	3.5	.35	−.456	.096
					Mean	.086

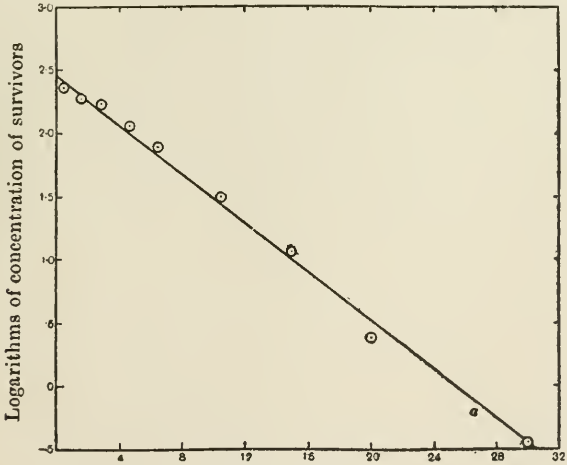

Fig. 2. Disinfection of *B. typhosus* with 0.6% phenol at 20°C. (*a*) Exp. 8. 1. '10, Table II.

In the case of *Staphylococcus pyogenes aureus* there was a short period (4 minutes in case of 0.6% phenol at 20C) at the beginning of disinfection, after which disinfection conformed to the usual type and proceeded as a "reaction of the first order" (see Table III and fig. 3).

TABLE III.

(H. C., 1910, Table VI)

Disinfection of Staphylococcus pyogenes aureus with 0.6% *phenol at* 20° C.

EXP. 6. 4. '09.

Time, minutes $=t$	Amount of sample taken, drops	Numbers counted on plates (agar)	Mean	Mean no. of bacteria present in 1 drop disinfection mixture, $=N$	$\text{Log}_{10}N$	$K = \frac{1}{t_n - t_0} \log \frac{N}{N_n}$
Control	1	1369 1218	1293	1293	3.112	—
1	1	1160 1120 1143	1141	1141	3.057	—
3	1	974 1064 1093	1044	1044	3.019	—
$4=t_0$	1	986 929 940	952	$952=N_0$	2.979	—
5	1	693 669 762	708	708	2.850	.129
6	2	1062 1048 1148	1086	543	2.735	.122
7	3	1253 1167 1187	1202	401	2.603	.125
8	3	728 731	729.5	243.2	2.386	.148
9	5	1113 912	1012.5	202.5	2.306	.135
10	5	761 691 890	780.7	156.1	2.193	.131
12	10	626 326 410	454	45.4	1.657	.165
15	10	220 216	218	21.8	1.338	.149

Mean .136

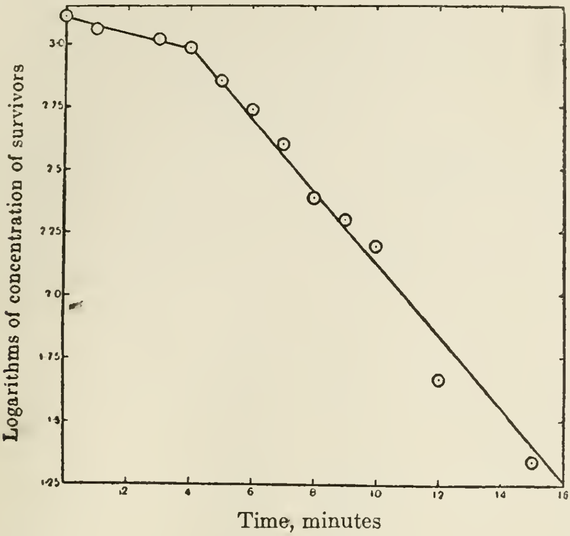

Fig. 3. Disinfection of *Staphylococcus pyogenes aureus* with 0.6% phenol at 20°C. (Exp. 6. 4. '09, Table III.)

Disinfection of *B. paratyphosus*, whether by metallic salts phenol, or other coal-tar derivatives was somewhat irregular; the reaction velocity fell off more rapidly as disinfection proceeded than it should have done if proportional only to the concentration of survivors. This point will be referred to later. (See p. 189.) The results of experiment with mercuric chloride are given in Table IV, as an example.

TABLE IV. (*Exp.* 23. 3. 07. H. C. 1908, Table X)

B. parat. Mercuric chloride, 2 per million. 20°C.

Time minutes	Mean no. of bacteria present in diluted sample		Mean no. of bacteria present in 1 drop disinfection mixture	K, assuming reaction to be in accordance with the equation $-\frac{dn}{dt}=Kn$
1.1	85	×250	21,250, taken as initial value of $n(=n_1)$ in calculating values of K	
2.1	82	×250	20,500	*
3	68	×250	17,000	*
4	52	×250	13,000	0.074
5	53	×250	13,250	0.050
7	62	×250	15,500	0.023
10	97	×125	12,125	0.027
15	96	×125	12,000	0.017
20	69	×100	6,900	0.026
30	139	×50	6,950	0.018
42	305	×25	7,625	0.011
51	279	×25	6,975	0.010
60	276	×10	2,760	0.015
80	215	×10	2,150	0.012
93	130	×10	1,300	0.013
140	—	—	150	0.015
180	—	—	49	0.015
210	—	—	29	0.013
245	—	—	29	0.012

* The values of n at these times are not sufficiently accurate to give consistent values for K.

Disinfection by hot water.

Disinfection by hot water proceeded as a "unimolecular" reaction in the case of *B. typhosus*, *B. coli commune*, and *B. pestis*. In the case of *Staphylococcus pyogenes aureus* and *B. paratyphosus* some irregularity was again displayed at the beginning of the disinfection, which afterwards proceeded in the usual manner. The results of two experiments with *B. coli commune* at two different temperatures are given below, as examples. (See Table V and fig. 4.)

TABLE V. (H. C. 1910., Table XII)

Disinfection of B. coli commune with hot water.

Exp. I, 15. 2. '10. Temperature 48.9°C.

Time, minutes $=t$	Amount of sample taken, drops	Numbers counted on plates (agar)	Mean	Mean no. of bacteria present in 1 drop disinfection mixture, $=N$	$\text{Log}_{10}N$	$K=\dfrac{1}{t_n-t_0}\log\dfrac{N_0}{N_n}$
$0.25=t_0$	1	371 362 383	372	$372=N_0$	2.570	—
1.0	1	266 253 304	274.3	274.3	2.438	.176
2	1	191 178 201	190	190	2.279	.166
4	1	68 76	—	68.25	1.834	.196
	2	129	—			
6	1	25	—	23	1.362	.210
	2	44	—			
10	2	16 29	—	12.4	1.093	.152
	5	67	—			
15	5	10	—	1.72	.235	.159
	10	15 18	—			
20	10	1 6	—	.425	−.372	.149
	20	10	—			
					Mean	.172

Exp. II, 15. 2. '10. Temperature 52.7°C.

$0.25=t_0$	1	416 331 402	383	$383=N_0$	2.583	—
1.0	1	207 235 183	208	208	2.318	.353
2	1	93 73 103	89.7	89.7	1.953	.360
4	2	21 47 19	29	14.5	1.161	.379
6	3	4 3	—	1.1	.041	.442
	5	5	—			
8	10	6 5	5.5	.55	−.260	.367
10	10	1 0 1	.66	.066	−1.181	.386
					Mean	.381

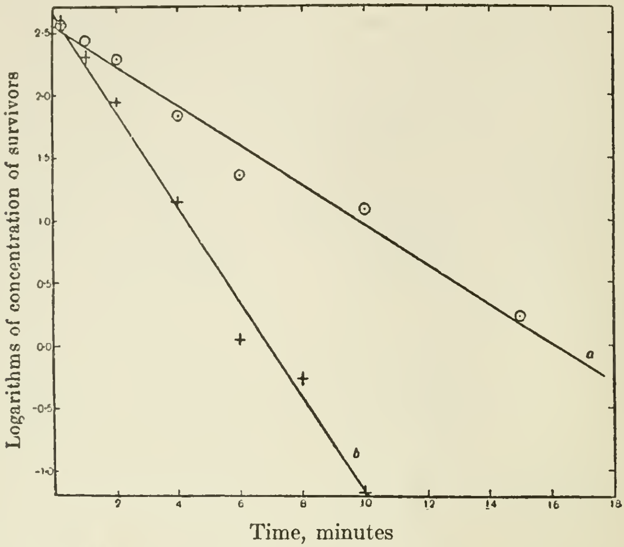

Fig. 4. Disinfection of *B. coli commune* with hot water (Table V).
(*a*) Exp. I at 48.9°C. (*b*) Exp. II at 52.7°C.

DISINFECTANT ACTION OF SUNLIGHT AND DRYING

Drying. Paul & Prall (1907) while investigating the suitability of the "garnet" method for disinfection experiments with staphylococcus, allowed garnets, coated with a thin dry film of bacteria to remain at various temperatures and counted the surviving bacteria from time to time. It was found that while the number of living organisms remained constant for many weeks at the temperature of liquid air, the numbers slowly decreased at ordinary temperatures. Paul (1909) has analysed the above data and has shown that death by drying also proceeds in accordance with a logarithmic law. (See also Paul, Birstein & Reuss, 1910.)

Sunlight. There are only very scanty experimental data upon this subject, but in the work of Clark & Gage (1903) one experiment is given with *B. coli* which gives consistent results and on analysis shows the same relationship.

The results are set out in Table VI and fig. 5, page 179. The concentration of survivors, determined from time to time by means of plate cultures; is seen to vary logarithmically with time, the value of the velocity constant, K remains fairly constant in value, and a straight line is obtained when logarithms of concentration of survivors are plotted against time, see Fig. 5.

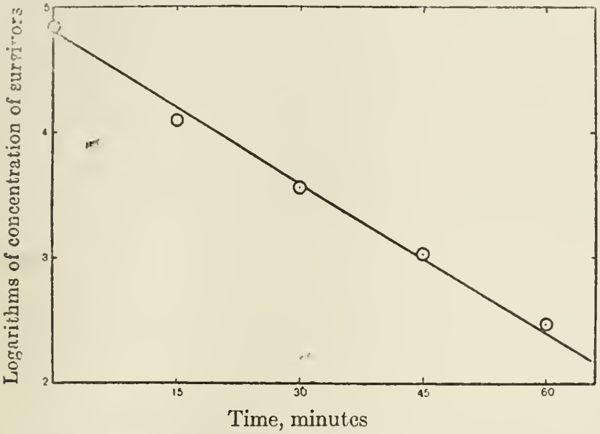

Fig. 5. Disinfection of *B. coli commune* by sunlight, results of Clark and Gage (1903), see Table VI.

TABLE VI.

Disinfectant action of sunlight upon B. coli commune.

(Clark and Gage, *Thirty-fourth Annual Report, State Board of Health, Massachusetts*, 1903.)

Time, minutes $=t$	Concentration of surviving bacteria, numbers per c.c. $=N$	$\log_{10}C$	$K=\frac{1}{t_n-t_0}\log\frac{N_0}{N_n}$
$0=t_0$	$71400=N_0$	4.854	—
15	12365	4.092	.051
30	3568	3.566	.043
45	1065	3.027	.041
60	295	2.470	.040
			Mean .044

It is not justifiable to draw a definite conclusion from such scanty data, but it is more than probable, judging from analogy, that the process of disinfection by sunlight will be found to fall in line with that experimentally established for other agents.

DISINFECTANT ACTION OF BLOOD-SERUM

In Table VII are given the results of a hitherto unpublished experiment with *B. colicommune* and normal rabbits' serum, at 30°C. After a latent period of about three and a half hours, disinfection proceeded slowly and in fair accordance with the logarithmic law (see values of the velocity constant in column 6).

TABLE VII

Disinfection of B. Coli commune with normal rabbits' serum at 30° C.

EXP. 28. 11. 1912.

Time, hours $=t$	Amount of sample taken, drops	Numbers counted on plates, (gelatine)	Mean no. of surviving bacteria in 1 drop serum $=N$	$\text{Log}_{10}N$	$K = \frac{1}{t_n - t_0} \log \frac{N_0}{N_n}$
3.6	1	816 810	$813 = N_0$	2.910	—
4.1	1	532 538	535	2.728	0.36
4.5	1	396 392	394	2.595	0.35
5.0	1	263 276	269	2.430	0.34
5.7	1	117 123	120	2.079	0.40
6.4	1	57 42	48.5	1.686	0.44
7.0	1	29 33	31	1.491	0.42
8.2	1	30 20	25	1.398	0.33
9.1	1	16 18	17	1.230	0.30
10.2	1 5	17 54	11.8	1.072	0.28
11.4	1 5	6 36	5.3	.724	0.28

SUGGESTED INTERPRETATION OF THE EXPERIMENTAL RESULTS

The foregoing examples may be taken as typical illustrations of the experimental data which is available. In the light of such evidence, disinfection must, in my opinion, be regarded as an orderly time-process presenting a close analogy with a chemical reaction, the bacteria and the disinfectant being regarded as the respective reagents.

The reaction proceeds according to some rule similar to the Mass Law so that if the disinfectant be present in large excess relative to the bacteria, disinfection proceeds in the same manner as a reaction of the first order, that is to say, as a unimolecular reaction, or, what amounts to the same thing, as a bimolecular reaction with one reagent in excess. The hydrolysis of cane sugar is an interesting parallel, viz.:—a union between water and sugar which nevertheless proceeds as a unimolecular reaction, for as ordinarily arranged the water is present in so great an excess that one may consider its concentration to remain constant and the reaction velocity to be determined only by the active mass of the second reagent, the sugar.

In the case of disinfection by hot water a complete analogy is presented with the heat coagulation of proteins, which in the ideal, uncomplicated case has been shown by Martin and the author (1910–1911) to be a "reaction of the first order." The analogy is so close, being maintained as regards the accelerating effect of acids and alkalies, and the value of the temperature coefficient, that it is impossible to resist the conclusion that the death of bacteria by hot water is associated with the irreversible change caused by "heat coagulation" of the constituent proteins.

The reason, therefore, why disinfection by any of these means is not sudden, but gradual, and moreover proceeds in agreement with this well-defined logarithmic law, must be sought in the same direction as in the case of the decomposition of arsenious hydride, the inversion of sugar and the heat coagulation of proteins, all of which processes proceed as "uni-molecular" reactions. The explanation offered by the chemist in these instances is that at any one time, only a proportion of the molecules are in a condition to undergo the dissociation or chemical union as the case may be. This *temporary* increase in susceptibility is believed to be occasioned by temporary differences in the internal energy of the molecules, which are distributed according to the law of chance. Accordingly the *proportion* of molecules, liable to attack at any moment, remains constant.

The process of disinfection may be explained on a similar hypothesis. Owing to internal fluctuating changes in the bacterial constituents, some individuals are liable to attack at one time, others at another, the total number of individuals vulnerable at any one moment being a constant proportion of the surviving population.

Such a view has not, however, received the support of some prominent bacteriologists who prefer a more vitalistic explanation. It has been common knowledge for many years that all individuals in a collection of apparently similar bacteria are not disinfected with equal ease, and this fact has been interpreted for example, by Bellei (1904) as an indication of differing degrees of resistance inherent in the bacteria, which can be accounted for by biological variation. More recently this view has received additional supporters among whom may be mentioned Eykman (1908), Hewlett (1909) and Reichel (1909, p. 152).

The following considerations form a powerful argument against their interpretation. Let us suppose that the progressive nature of the disinfection process may be attributed to *permanent* differences of resistance occurring among the various individual bacteria. If such were the case disinfection would certainly be gradual and not sudden, but the rate at any time would only be proportional to the concentration of survivors if the different resistances were allotted according to one definite arrangement, an arrangement which could only occur as a remote coincidence, and could hardly be universally present. For, suppose that the disinfection of 100,000 bacteria is in question, that the survivors vary logarithmically with time, as in the ideal case of disinfection, and that for the sake of argument the rate happens to be a reduction to 1/10th in each minute,

10,000 = 1/10 of 100,000, survive after 1 minute,
1,000 = 1/10 of 10,000, will survive after 2 minutes, and
100 = 1/10 of 1000, will survive after 3 minutes,
10 = 1/10 of 100, will survive after 4 minutes, and so on

If the cause of this is to be sought in the possession by the bacteria of different individual resistances to disinfection we should have to suppose that these resistances had the following distribution among the 100,000 bacteria, viz. that

90,000 take 1 minute to kill,
9,000 take 2 minutes to kill,
900 take 3 minutes to kill,
90 take 4 minutes to kill, and so on.

Or, in the general case, if N bacteria die logarithmically, and if 1/x of N survive 1 minute, then

$1/x^2N$ will survive 2 minutes,
$1/x^3N$ will survive 3 minutes, and so on,

and the resistance of the various individuals will be so distributed that there are present

$$N - \frac{N}{x}, \text{ or } N\frac{(x-1)}{x}, \text{ which are killed in 1 minute,}$$

$$N\left(\frac{x^2-1}{x^2} - \frac{x-1}{x}\right), \text{ or } N\frac{(x-1)}{x^2}, \text{ which take 2 minutes to kill,}$$

$$N\left(\frac{x^3-1}{x^3} - \frac{x^2-1}{x^2} - \frac{x-1}{x}\right), \text{ or } N\frac{(x-1)}{x^3}, \text{ which take 3 minutes to kill,}$$

and no other distribution will satisfy the necessary condition.

A further argument can be brought ~~against a theory of variable~~ permanent resistance, viz., that biological characteristic is as a rule distributed in a different manner. Those individuals possessing it in moderate amount are usually in the greatest number, while those possessing it in greater or less degree are in the minority. In the case of disinfection, those apparently possessing the least resistance are invariably in the greatest number. a variable

Reichenbach (1911) admits the reasoning contained in the preceding paragraph, but has in an ingenious manner attempted to show that such a definite grading of resistances might nevertheless, under certain circumstances, be produced in a bacterial population owing to the peculiar[1] way in which this has its origin,

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[1] Reichenbach's argument loses weight when it is remembered that the death of some higher organisms (Chlamydomonas, Harvey, Annals of Botany, vol. XXIII, p. 181, 1909; seeds of barley, Darwin and Blackman, British Associa- b

i. e. by continued subdivision of the existing organisms. This multiplication at first is general, but as time goes on is maintained by a decreasing proportion of the bacteria. Two assumptions are made, first, that the age of a bacterium and its resistance are bound up with one another and secondly, that the number remaining undivided in each successive generation increases with mathematical regularity.

Reichenbach feels forced to adopt some such explanation for the following reason. He considers it unlawful to extend an argument, based on chemical analogy, from a conceivable logarithmic destruction of constituent molecules of the bacterial cell, to explain the logarithmic death of the bacteria themselves. Taking the relative size of molecule and bacterium into account it is true that if the death of the latter occurred only after changes had taken place in a large number of constituent molecules, we might regard the various individual organisms as a series of similar vessels in which the same chemical reaction was running its course. If, in all, the conditions were exactly alike the reaction would in all cases arrive at the same stage at equal times. If then the death of a bacterium (or its inability to multiply further, which in all this work is taken as the criterion of death) is considered to be occasioned by a certain degree of internal destruction, a collection of similar individuals would
ex all die after the same period of ~~re~~posure to a disinfectant.

It must be admitted that the above argument is a real difficulty in the point of view I wish to advocate. In order to meet it one must assume that the constituent of the bacterium which is essentially concerned must be present in very small amount and that the number of its units are commensurate with the number of bacteria present, so that the bacteria themselves may be regarded as units in the process without gross inaccuracy.

In contradistinction to these theories of graded resistances, I would therefore explain the logarithmic nature of the dis-

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tion, Dublin Meeting, 1909), under influence of poisons and hot water, also proceeds so that death-rate remains proportional to the concentration of survivors.

infection process as due to an essential *similarity* among the individual bacteria. For this view there is the following experimental support. It has been found that any circumstances which may be reasonably supposed to conduce to a more perfect uniformity among the individual bacteria, do not disturb the logarithmic precision of their disinfection, but make it the more perfect. On the other hand, where a departure from the law has been noticed, the cause has been traced to permanent dissimilarity among the units of the bacterial population.

The micro-organisms whose disinfection proceeds in most accurate agreement with the "unimolecular type" are the spores of *B. anthracis* whether suffering death by phenol mercuric chloride or hot water. In these cases the material had previously been heated to a high temperature (80°C.) in order to kill any vegetative forms which might have been present. Incidentally any weakly spores also perished and the resulting collection of organisms possessed greater uniformity as regards resistance.

Disinfection of *B. paratyphosus* illustrates the converse agrument.

In the case of 24 hours' cultures the rate of disinfection, whether by phenol or hot water, fell off more rapidly as disinfection proceeded than it should have done if proportional only to the concentration of surviving bacteria. This discrepancy was minimised and disinfection proceeded in nearer agreement with the ideal type, if experiments were made with material obtained by successively sub-culturing from broth to broth every 2–3 hours. (See fig. 6.)

It is probable from the researches of Lane-Claypon (1909) and others that during the earlier hours of its life multiplication is very general among the population of a bacterial culture at 37°C.; later on the proportion of organisms remaining undivided becomes greater and greater as the concentration of bacteria approaches the limit which the medium can sustain. In a young culture therefore (sown also from a young culture to avoid admixture of veterans) the entire population will be of approximately the same age and generation. In a 24 hours' culture on the other hand there would obviously be less uniformity in this respect. It is very significant therefore that the former material

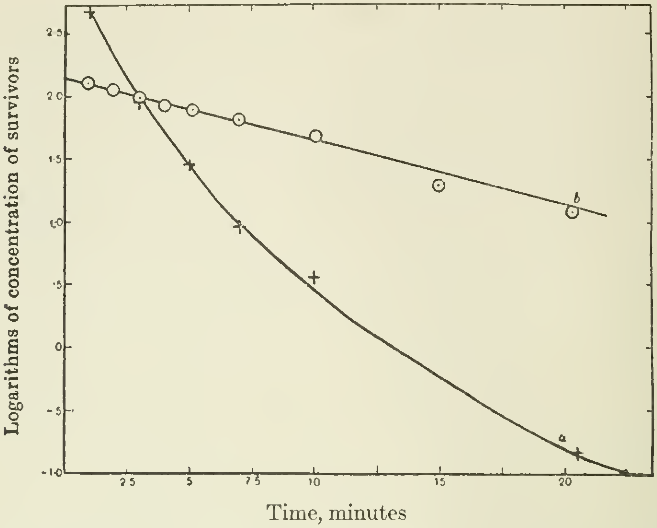

Fig. 6. (*a*) Disinfection of 24 hours' culture of *B. paratyphosus* with 0.6% phenol at 21°C. (H. C. 1908, Table VII.)

(*b*) Disinfection of 3 hours' culture (3rd generation of 3 hours' cultures) of *B. paratyphosus* with 0.6% phenol at 20°C. (H. C. 1908, Table XII.)

in disinfection should approximate more nearly to the ideal case.

The above interpretation of the facts of disinfection on the lines of chemical analogy has also received support from experimental data showing the influence upon reaction velocity of variation in

(a) Concentration of Disinfectant.
(b) Temperature.

(*a*) *Concentration of Disinfectant*

The ideal method of obtaining this information is by comparison of velocity-constants with varying concentration of disinfectant. This was, however, rejected owing to the extremely laborious nature of the necessary experiments, and an "end-point" method was substituted. The time was measured which was necessary for (almost) complete disinfection of a constant number of bacteria in various strengths of disinfectant at constant temperature; this time was taken to be inversely proportional to the mean reaction—velocity

of the process. In most cases about 30,000,000 bacteria were added to 5 c.c. disinfection mixture and samples (=0.08 c.c.) removed from time to time and tested for surviving bacteria by means of liquid sub-cultures. Disinfection-time was taken to be the period elapsing before a negative test-culture was obtained, strictly, the time taken for reduction of about 5 million organisms per c.c. to less than ~~80~~ per c.c. (*i.e.* when there is not one living bacterium in 12
0·08 c.c.).

A definite logarithmic relationship between velocity of disinfection and concentration of disinfectant was found to exist in all the cases investigated.

If the two variables were plotted one against the other the experimental points lay on regular curves. Fig. 7 below gives the results of disinfection by phenol of *B. paratyphosus* and *Staphylococcus pyogenes aureus* and may be taken as typical of a series of similar experiments.

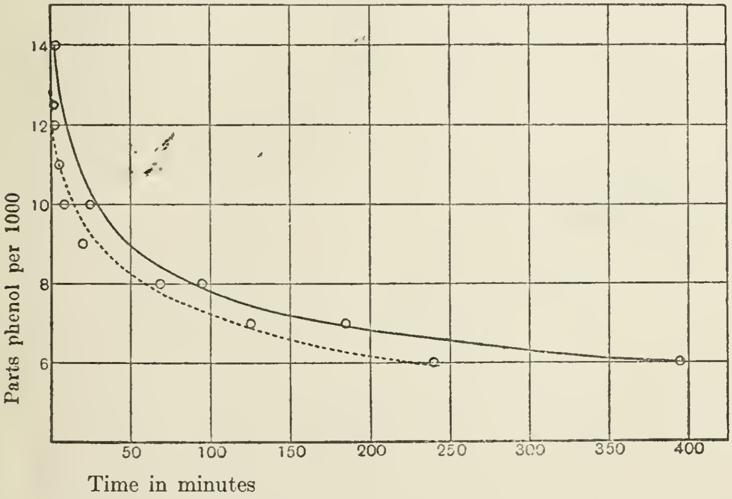

Fig. 7. Effect of varying the concentration of phenol upon the time taken for disinfection of (*a*) *B. paratyphosus*, dotted curve (H. C. 1908 Exp. 21. 6. 06, Table XVI): (*b*) *Staph. aureus*, continuous curve (Table XVII).

Watson (1908) has examined all the experimental data and showed that this logarithmic relationship may be expressed in the form

$$C^n t = \text{a constant},$$

or $\log C$ ~~and~~ $+$ $\log t =$ a constant, where $C =$ concentration of disinfectant and $t =$ time taken for disinfection.

TABLE VIII (H. C. 1908 Table XVI)

Phenol. *B. paratyphosus.* 20° C. 5 drops (.085 c.c.) of a 24 hours' broth culture (about 30,000,000 bacteria) added to 5 c.c. disinfectant solution.*

	Parts phenol per 1000 $= C$	Time taken for disinfection, *i.e.*, reduction in number of about 30,000,000 individuals to less than 60 $= t$	$5.5 \log C + \log t$†
Exp. 6. 6. 06	8	45 minutes	6.62
	7.5	75	6.69
	7	105	6.67
	6.5	125	6.58
	6	225	6.64
	5.5	440	6.71
	5	11.5 hours	6.68

* These and all subsequent values of the expression are calculated with Briggs' logarithms.

† See Watson (1908) Table I.

TABLE IX (H. C. 1908 Table XVII)

Phenol. *Staphylococcus pyogenes aureus.* 20° C.

Phenol parts per 1000 $= C$	Time taken for disinfection $= t$	$5.5 \log C + \log t$*
14	4.5 minutes	6.95
12.5	2.5	6.42
10	25	6.90
8	95	6.94
7	186	6.92
6	395	6.88
4	23.7 hours	6.47

* See Watson (1908) Table II.

He has also pointed out that this is the relation which would hold for a chemical reaction where one molecule of one substance reacts with N molecules of a second, the latter being in great excess.

Tables VIII and IX show the value of the constant ~~M~~ in the N two experiments with phenol set forth in fig. 7. It is noteworthy that the action of phenol upon the two organisms appears to be of the same character, the value of N being equal to 5.5 in either case. This indicates, according to Watson, that in the disinfection of *B. paratyphosus* or *Staphylococcus py. aur.* with phenol with the molecules of phenol reacting those of the bacterial constituent are in the proportion of 5.5 to 1.[1]

As regards metallic salts, the same law held good for disinfection by silver nitrate with a value of N, for *B. paratyphosus*, equal to 0.89. In the case of mercuric chloride, however, the above logarithmic relation between the concentration of disinfectant and the average velocity of disinfection is maintained only if the former is expressed in terms of the corresponding concentration of mercuric ions. Under these circumstances N has the value of 4.9 for anthrax spores (data of Krönig and Paul) and 3.8 for *B. paratyphosus*. This comprises the conclusion arrived at by Krönig and Paul that, in disinfection by metallic salts, the metallic ion is the real disinfecting agent. In the case of silver nitrate, electrolytic dissociation is so nearly complete in dilute solution, that the concentration of silver ions are in proportion to the concentration of the salt.

In these instances there is some evidence that disinfection is accompanied by a normal union of the metallic salt with the bacterial protein. A centrifuged deposit of bacteria previously treated with $HgCl_2$ although well washed, shows marked blackening on addition of SH_2 water.

Whether Watson's somewhat bold interpretation be correct or not, there seems to be no doubt that, as far as the available data go, the relation of average reaction-velocity, V_1, of disinfection to concentration of disinfectant is expressed by the formula

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[1] The matter would appear to be somewhat more complicated. The above may be true of the ultimate disinfection process. But it is probable from the researches of Reichel (1909) and Cooper (1912) that the first step in disinfection by phenol and other coal tar derivatives is a partition of the disinfectant between the water and the bacteria.

$$C^n = KV. \text{ or}$$

$C^n t = K_2$ (where t, time of (almost) complete disinfection, is taken as proportional to the reciprocal of the average velocity of disinfection).

(*b*) *Temperature*

The results of a fairly comprehensive series of experiments show that the reaction velocity of disinfection is influenced by temperature in an orderly and consistent manner, in accordance with the law of Arrhenius. As regards the *magnitude* of the effect, disinfectants fall into two classes (1) those in which disinfection is accelerated by rise of temperature to a degree commensurate with that of most chemical reactions, *i.e.*, 2 to 4-fold for a rise of 10° C. and (2) those in which the effect of temperature is much greater.

Disinfection with mercuric chloride and silver nitrate fall into the first category.[1] Madsen and Nyman (1907) found the temperature coefficient for anthrax spores to be 2.5 for 10° C. rise in temperature over a range from 25° to 45° C. In Table X, which gives the results of two experiments with mercuric chloride and *B. paratyphosus* average disinfection-rate is seen to be accelerated about 3.5-fold for a rise in temperature of 10° C. between the temperatures of 0° and 42° C. The value of the constant $A = \frac{T_0 T_n}{T_0 - T_n} \log \frac{t_n}{t_0}$ (where t_n t_0 = times of disinfection (reciprocals of average velocity of disinfection) at the absolute temperatures T_n and T_0 respectively) in the 4th column of Table X shows the degree of approximation to the law of Arrhenius.

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[1] Disinfection by drying has also a low temperature coefficient viz.:—2–3 for 10° C. (Paul, 1909, Paul Birstein & Reuss, 1910).

TABLE X (H. C. 1908 Tables XXVII and XXVIII)

Effect of temperature upon disinfection of B. paratyphosus with mercuric chloride

	Concentration of $HgCl_2$, parts per 1000	Temperature degrees centigrade	Time taken for disinfection†	Values of A $\left(=\frac{T_0T_n}{T_0-T_n}\log\frac{t_2}{t_1}\right)$*, where initial absolute temperature $T_0=293$, and initial time $t_0=2.5$	Relative increase in mean reaction—velocity for 10° C. rise in temperature
Exp. I. 9. iii. 07	1.0	42	<0.5 min.	—	
		29.7	<0.5	—	
		20	2.5	—	
		12.6	4.5	2890	
		7.9	6	2590	
		0	41.5	4880	
				Mean = 3420	3.9
				$T_0=314.6$	
				$t_0=0.75$	
Exp. II. 6. iii. 07	0.1	41.6	0.75	—	
		30.7	2.5	4580	
		19.8	11.5	5010	
		13.9	36	5480	
		6.8	50	4610	
		0	101	4390	
				Mean = 4810	3.0

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* These and all subsequent values of this expression are calculated with Briggs' logarithms.

† Strictly speaking, reduction of about 6,000,000 bacteria per c.c. to less than about 12 per c.c.

In the case of phenol and other coal-tar derivatives the effect of temperature is much more marked. Average velocity of disinfection was increased in the case of *B. paratyphosus* and phenol from 7-fold to 15-fold for a rise of 10° C., according to the strength of the disinfectant. The results of two experiments are given below in Table XI to serve as types. Some idea of the capacity of this kind of work to yield accurate results, in other words of the possibility of obtaining constant conditions of experiment, may be gleaned from the fact that it was possible to fill up gaps in one experiment with results obtained on two other occasions.

TABLE XI (H. C. 1908 Tables XXXIII and XXXV)

Effect of temperature upon disinfection of B. paratyphosus with phenol

	Concentration of phenol, parts per 1000	Temperature degrees centigrade	Time taken for disinfection	Value of A $\left(=\frac{T_0 T_n}{T_0 - T_n}\log\frac{t_n}{t_0}\right)$, where initial abs. temp. $T_0 = 308.7$ and initial time $t_0 = 9$	Relative increase in average reaction—velocity for 10° C. rise in temperature
EXP. 27. ix. 06	6.0	44	<1 minute	—	
		35.7	9 minutes	—	
		30.5	27.5	8650	
EXP. I. x. 06		28	47.5	8660	
		25	95	8750	
		21	251	8890	
		16	350	7180	
		6	more than 10 hours	—	
				Mean = 8430	7.2
				initial abs. temp. $T_0 = 303.5$ and initial time $t_0 = 1.5$	
EXP. 24. ix. 06	10.0	30.5	1.5 minute	—	
		27.5	3.5 minutes	*	
		21.3	9	8090	
		16.5	27	7870	
EXP. 26. ix. 06		15.8	23.5	7130	
		6	226	7530	
EXP. 25. x. 06		0	17.5 hours	7910	
				Mean = 7710	7.5

* Figure obtained was not concordant.

† Strictly speaking, reduction of about 6,000,000 bacteria per c.c. to less than about 12 per c.c.

The temperature effect is of a still higher order of magnitude in disinfection by hot water. The velocity of disinfection is increased more than 100-fold per 10°C rise of temperature in case of *B. typhosus*, 29-fold per 10°C for *Staphylococcus pyogenes aureus*, 12-fold per 10°C for *B. coli*. The details of some experiments with *B. typhosus* are given in Table XII below, and in fig. 8 the logarithmic relation between disinfection-rate (reciprocal of times taken for disinfection) and the temperature of experiment is shown graphically.

TABLE XII (H. C. 1910. Table XXI)

Temperature coefficient of disinfection of B. typhosus with hot water (end-point method)

	Temp. of exp., °C.	Time taken for disinfection,* minutes=t	Log$_{10}t$	Log$_{10}$ (temperature coefficient for 1°C.)	Temperature coefficient per 1°C.	Temperature coefficient per 1°C.
Exp. I, 10. 6. '09.	59	1.26	.100			
	54	22.5	1.352	.250	1.78	
	49	196	2.292	.188	1.54	
Exp. II, 4. 6. '09	57	7.5	.875			
	52.1	53	1.724	.173	1.46	
					Mean 1.60	110

* Strictly speaking, reduction of about 5,000,000 per c.c. to less than about 12 per c.c.

A high temperature coefficient is characteristic of many reactions involving bodies of a protein nature (Famulener & Madsen, 1908; Madsen & Streng, 1909), including the coagulation of proteins by hot water, which has been found by Martin and the Author (1910) to be accelerated from 14 to 650 times. Hartridge (1912) finds the temperature coefficient for "heat coagulation" of alkaline methaemoglobin to be as great as 725 for 10°C rise in temperature in case of oxyhaemoglobin and egg-albumen respectively if the temperature is raised 10°C.

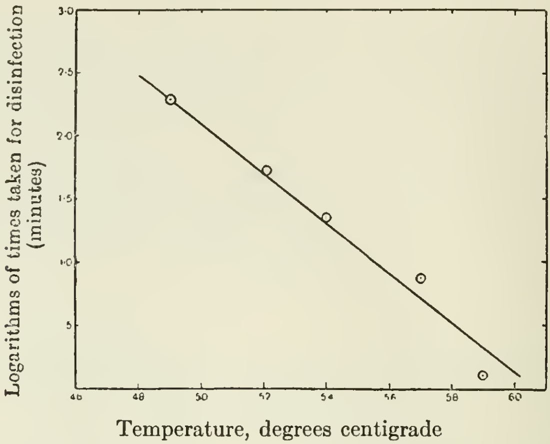

Fig. 8. Effect of different temperatures upon disinfection of *B. typhosus* by hot water (Table XII).

The logarithmic nature of the process in both coagulation of proteins and disinfection of bacteria by hot water, combined with the similar magnitude of the temperature coefficient indicates that the latter process is concerned with heat-coagulation of the constituent proteins. Additional confirmation is obtained from the exactly analogous effect of traces of acid and alkali in the two instances.

General Conclusions

The facts brought to light by the use of quantitative methods of study have shown disinfection in the ideal, uncomplicated, case to be an orderly time-process which is so arranged that the rate of disinfection at any moment is proportional to the concentration of surviving bacteria. This, in my opinion, can be most adequately explained by assuming an inherent similarity to exist between the various individuals so that their death will occur in accordance with the laws of chance, in other words a constant proportion of the surviving population will be killed in unit time. Yule (1910) has shown that if one condition is necessary for death, as a multitude of small causes, among a number of similar individuals, death will occur logarithmically, if the result is governed by the law of probability.

In order to obtain a mental picture of the *way in which* these chance causes of death may operate, attention is naturally directed to the consideration of other processes which take place in a similar manner, viz.:—chemical reactions "of the first order" such as the decomposition of hydrogen arsenide, the inversion of sugar or the "heat coagulation" of proteins. The closest possible analogy has been demonstrated between the last instance and the destruction of bacteria by hot water.

In these cases an explanation has been sought in temporary changes in the energy of the molecules as a result of which all the molecules are not equally sensitive to attack at the same moment. Similarly, temporary changes in resistance are attributed to otherwise similar bacteria whereby at a given moment only a certain number will be vulnerable, that number being proportional to the total number of individuals surviving in unit volume.

Disinfection, whether by disinfectants, heat or other agencies, exhibits the closest analogies with a chemical reaction, the velocity of which is controlled by external conditions, such as temperature or the concentration of bacteria and disinfectant.

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ON THE "HEAT COAGULATION" OF PROTEINS. PART III. THE INFLUENCE OF ALKALI UPON REACTION VELOCITY.

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We have pointed out in the papers we have previously published upon the subject ((1) and (2)) that the "heat coagulation" of proteins as ordinarily observed consists of two distinct processes (1) "denaturation[1]," or the reaction between protein and hot water, and (2) agglutination, or the separation of the altered protein in a particulate form.

In our previous work we investigated the first process using solutions of pure hæmoglobin and egg-albumen containing a small proportion of ammonium sulphate (0·3 % to 0·4 %) and possessing different slight degrees of acidity. In these cases agglutination occurred at a rate very greatly in excess of denaturation; the latter process thus became the limiting factor in the complete reaction and the reaction-rate could therefore be studied without complication. The reaction velocity of denaturation was found to be increased with the amount of free acid present. Since the latter was decreased continuously with the gradual precipitation of the protein, the course of the reaction departed from that followed by a "reaction of the first order" to which type it conformed if means were taken to maintain constant acidity during the process ((2), p. 5).

In the present investigation we have studied heat coagulation in the presence of free alkali.

The material used was again recrystallised egg-albumen from which the ammonium sulphate had been removed by dialysis. Solutions were made, all containing 1 % protein and various concentrations of free alkali, by addition of a standard solution of sodium hydroxide. A control experiment was made with the solution in its normal reaction, which was slightly acid (hydrogen-ion concentration equal to 10^{-5} normal). The method of experiment was similar to that described in our first paper ((1), p. 407), viz. :—estimation of the residual protein at successive intervals of time. In this case, however, the conditions obtaining in the solution were such as prevented the agglutination of the denaturated protein. It was therefore necessary to adjust the reaction and salt-content of the cooled samples (see below) so that the altered protein should be precipitated. The temperature selected was 64·5° C.; all the solutions were warmed to 20° C. before placing in the thermostat and eight minutes' interval was allowed for the tube and contents to attain the required temperature, before the first sample was removed.

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[1] We have adopted the word "denaturation" as an English equivalent of "Denaturierung" which is in general use in German to denote the first process in the precipitation of proteins by hot water.

The samples were quite unchanged in appearance but, on acidifying, more or less precipitation occurred according to the time during which the solution had been heated.

It has been shown that for satisfactory precipitation of heated proteins a definite, slightly acid, reaction is necessary. The optimum acidity for agglutination of denaturated egg-white was found by Sörensen and Jürgensen(3) to be 2×10^{-5} normal and that of denaturated serum-proteins was previously found by Michaelis and Rona(4) to be $0·3 \times 10^{-5}$ normal, and to coincide with the iso-electric point of the proteins. In the presence of electrolytes these conditions may become very much modified. We have found, for example, that at 69° C. denaturated, pure egg-albumen in the presence of a small concentration of ammonium sulphate agglutinated over a range of hydrogen-ion concentration extending from $10^{-4·6}$ ($2·5 \times 10^{-5}$) normal to $10^{-7·4}$ ($·004 \times 10^{-5}$) normal ((1), pp. 421 and 423).

In order to bring about agglutination the following procedure was adopted: the samples were acidified by the addition of 1/10th normal acetic acid in slight excess of the amount of alkali originally added, and enough pure sodium chloride was then added to saturate[1] the solution. After standing for 24 hours, the coagulum on boiling was determined in a measured volume of the filtrate. In calculating the concentration of unaltered albumen in the original sample, due allowance was made for dilution of the protein-content by addition of acetic acid and saturation with sodium chloride. In the control (Exp. 1, Table I) agglutination occurred naturally, but the samples were nevertheless saturated with sodium chloride for the sake of uniformity.

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[1] This was found to be a useful method of securing complete precipitation of denaturated protein in cases where the reaction was not correctly adjusted to be at the best point for agglutination.

The "alkalinity" of the various solutions was also investigated. Direct determinations of the hydrogen-ion concentration were made electrically, using the apparatus recommended by Michaelis and Rona(5), which we have found to be specially suitable for small quantities of liquid. In their method the hydrogen electrodes are maintained in a still atmosphere of hydrogen, and equilibrium is attained almost instantaneously[1] if the electrodes are dipped into their respective solutions to a depth of 1 to 2 mm. only. Contact between the standard cell

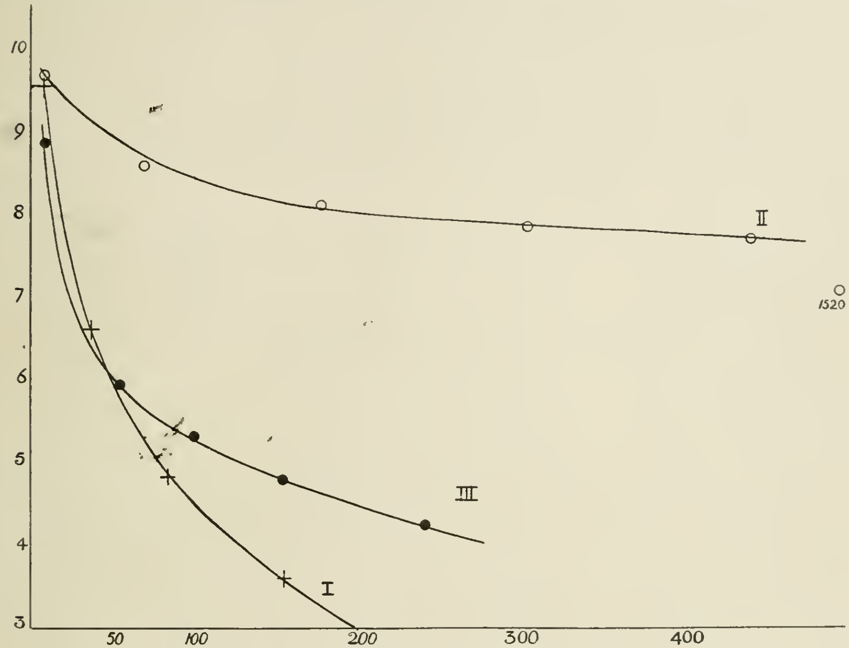

Fig. 1. Denaturation of crystalline egg-albumen, 1 °/₀ solution, at 64·5° C.
(I) In original (faintly acid) reaction.
(II) After addition of 5·0 c.c. N/10 NaOH per gram protein.
(III) ,, ,, 10·0 ,, ,, ,, ,,
Ordinates = concentration of residual albumen in milligrams per c.c.
Abscissæ = time in minutes.

---

[1] It is necessary to make a reading as soon as possible after completing the circuit, because with the use of concentrated potassium chloride in the contact thread there is a tendency for fluid to soak up into the thread from either cell and dilute the salt; a contact potential is thereby developed and the electromotive force in the circuit falls.

containing 1/10th normal HCl and that containing the solution under investigation was made by means of a concentrated solution of potassium chloride (3·5 normal). The contact potential was found to be nearly obliterated by this means and as the electromotive force measured was comparatively large (usually more than 0·5 volt) no correction was applied. Concentration of hydroxyl-ions was calculated from the hydrogen-ion concentration thus determined, taking the dissociation constant of water to be equal to $10^{-14.14}$ (Sörensen (6), p. 161).

The results of the experiments are given in Table I, and graphically expressed in Fig. 1. In Exp. 2, where the reaction was on the alkaline

TABLE I. *Coagulation of dialysed crystalline egg-albumen,* 1·03 % *solution, at* 64·5° C., *in natural reaction (acid) and after addition of sufficient amount of sodium hydroxide to make the solution alkaline in reaction.*

Exp.	$\frac{N}{10}$ NaOH added per grm. protein, c.c.	Time, minutes	Concentration* of residual albumen, mgrs. per c.c.	Concentration of H+ in terms of normality	Calculated concentration of OH− in terms of normality†	Time taken for reduction of concentration of residual albumen from 9 mgs. per c.c. to 7·12 mgs. per c.c., minutes
1	0	0 = control	10·30	$10^{-4.91}$ normal (124×10^{-7} normal)	—	18·5
		8	9·61	—	—	
		37	6·64	—	—	
		83	4·85	—	—	
		154	3·61	$10^{-5.48}$ normal (33×10^{-7} normal)	—	
2	5·0	0 = control	10·30	$10^{-10.36}$ normal	$10^{-3.78}$ normal (1.67×10^{-4} normal)	14/72 1472
		8	9·73	—	—	
		69	8·63	—	—	
		177	8·15	—	—	
		303	7·89	—	—	
		440	7·75	$10^{-9.43}$ normal	$10^{-4.71}$ normal (0.19×10^{-4} normal)	
		1520	7·12	$10^{-8.90}$ normal	$10^{-5.24}$ normal (0.058×10^{-4} normal)	
3	10·0	0 = control	10·30	$10^{-11.40}$ normal	$10^{-2.74}$ normal (18×10^{-4} normal)	13·5
		8	8·91	—	—	
		54	5·96	—	—	
		99	5·33	—	—	
		153	4·80	—	—	
		240	4·25	$10^{-11.12}$ normal	$10^{-3.02}$ normal (9.6×10^{-4} normal)	

* Corrected for dilution by saturation with NaCl and also by addition of N/10 acetic acid in case of experiments 2 and 3.

† Dissociation constant of water being taken as $10^{-14.14}$.

side of the neutral point (hydroxyl-ion concentration equal to $10^{-3\cdot8}$ normal), denaturation proceeded so slowly at 64·5° C. that after more than 24 hours had elapsed, only 30 % of the total protein was altered by heat. On addition of more alkali (Exp. 3, hydroxyl-ion concentration equal to $10^{-2\cdot7}$ normal) the denaturation-rate had increased so as to be comparable with that in the original, slightly acid, solution (Exp. 1).

The progress of the reaction in alkaline solutions conforms to no simple law. The reaction-velocity does not remain proportional to the concentration of residual protein, but departs more and more from the theoretical value as the experiment proceeds. This is due to the progressive diminution in alkalinity (see Exp. 2) which is taking place in the solution at the same time. This change in reaction is exactly comparable with the progressive diminution of acidity during coagulation in acid solutions (see Exp. 1, and our previous paper, (2), Tables III and IV, p. 8).

The denaturation process when alkalinity is kept constant. An attempt was made to keep "alkalinity" constant during the process of denaturation by means of the presence of excess of solid magnesium oxide, a saturated solution of which possesses a hydroxyl-ion concentration of about $10^{-4\cdot0}$ normal. A solution of pure egg-albumen, containing about 0·5 % ammonium sulphate, was shaken up with excess of magnesium oxide and left for 24 hours. The solution was gradually warmed up to 60° C., so that loss of equilibrium owing to changed solubility of magnesium oxide at the higher temperature might be avoided, and then placed in the thermostat at 65° C., at which temperature denaturation proceeded at a convenient rate. The initial concentration of egg-albumen was about 0·9 % and the residual protein after the various intervals of time was determined in the usual way.

TABLE II. *Coagulation of pure egg-albumen solution in presence of excess of magnesium oxide at 64·8° C.*

Time, minutes $= t$	Residual albumen, mgs. per c.c. $= C$	$\mathrm{Log}_{10}\, C$	$K = \frac{1}{t_n - t_0}(\log C_0 - \log C_n)$	Concentration of OH^- ions, in terms of normality
10	—	—	—	$10^{-4\cdot14}$ normal ($7\cdot3 \times 10^{-5}$ normal)
$41 = t_0$	$8\cdot676 = C_0$	·938	—	—
102	—	—	—	$10^{-4\cdot16}$ normal ($6\cdot8 \times 10^{-5}$ normal)
162	8·032	·905	·00028	—
312	7·100	·851	·00032	$10^{-4\cdot32}$ normal ($4\cdot8 \times 10^{-5}$ normal)
1380	3·880	·589	·00026	—

Even in the presence of excess of magnesium oxide, the concentration of hydroxyl-ions was not maintained absolutely constant and was reduced during the experiment to 70 % of its original value (see Table II). This reduction is small compared with what occurred in the other experiments in which no such precautions were taken (Table I). Having nearly maintained constancy in hydroxyl-ion concentration denaturation proceeded in fair agreement with the unimolecular law, and the velocity constant, K (column IV), remained reasonably constant in value.

It was found that in the presence of magnesium oxide the denaturated protein was almost completely agglutinated in the alkaline solution and little further was precipitated on acidification This is to be ascribed to the influence of the strongly electro-positive Mg^{++} upon the negatively charged protein particles.

Influence of hydroxyl-ion concentration upon denaturation velocity. Since no direct comparison of velocity constants was possible in the case of Exps. 1, 2 and 3 (Table I), the rates of denaturation were compared by considering the times taken in the different instances for denaturation of exactly similar proportions of the original egg-albumen present. This was done by means of the curves in Fig. 1, and the numbers obtained are given in the last column of Table I. Unfortunately it was only possible to institute a comparison over a small portion of the curves, because of the slow rate in Exp. 2, but the figures obtained are very striking. Increase of original alkalinity from a concentration of hydroxyl-ions equal to $10^{-3.8}$ to $10^{-2.7}$ was accompanied by an increase in average denaturation-rate of over a hundredfold.

In a previous paper we showed (II), Tables V and VI, pp. 419 and 421) that at 69° C. coagulation-rate progressively decreased as the original acidity (hydrogen-ion concentration equal to $10^{-4.60}$ normal) was neutralised by addition of ammonia. These experiments were carried to the neighbourhood of the neutral point (hydrogen-ion concentration equal to 10^{-7} normal), but before this was reached the changes in reaction-velocity became less marked, the absolute differences in hydrogen-ion concentration being also inconsiderable when compared with those existing between the more acid solutions. It would therefore appear that in the neighbourhood of the neutral point change in hydrogen- or hydroxyl-ion concentration is less effective in modifying denaturation-rate than in regions further removed.

There are other instances of the influence of acid and alkali on the effect of hot water upon protein bodies which offer an analogy with what we have found to obtain in the case of egg-albumen. Famulener and Madsen(7) showed that the destruction by hot water of the activity of the antigens, vibriolysin, tetanolysin, and goat-serum hæmolysin, proceeded as a reaction of the "first order," that the reaction-velocity was at a minimum when the solution was neutral in reaction and was greatly and progressively increased on addition of either acid or alkali. Streng(8) showed that the rate of destruction by hot water of "coli-agglutinin" was greatly increased by addition of alkali. He also showed that while in some cases the reaction conformed to the "monomolecular type," in many others, including those solutions to which alkali had been added (p. 96), the reaction-velocity during the progress of the reaction became progressively lower than the value it should have maintained if it had remained proportional to the concentration of unchanged agglutinin. We have shown ((1), p. 408) that the coagulation of crystalline oxyhæmoglobin by hot water proceeds as a reaction of the first order, and Hartridge(9), who has confirmed our results with oxyhæmoglobin and has shown the same to be true for alkaline methæmoglobin, finds that in the latter case the reaction-rate greatly exceeds that of hæmoglobin at the same temperature. One of us (H. C.(10))[1] showed that the death-rate of a suspension "of bacteria in" hot water, which also proceeds as a reaction of the unimolecular type, was accelerated to a surprising extent by the addition of small quantities of either acid or alkali to the distilled water in which they were heated.

Famulener and Madsen regarded the above accelerating effect of acids and alkalies as an example of catalysis. We should prefer to explain it as due to a difference in properties exhibited by protein according to whether it exists alone or in combination with acid or alkali as a salt. We are of opinion that the protein salts are denaturated at a greater rate than the amphoteric protein at the same temperature, and that the rate of denaturation depends upon the degree of salt formation which has taken place.

There are a number of observations suggesting that protein salts have a greater attraction for water than electrically neutral proteins. Pauli and Handovsky(11) and Schorr(12) have shown that proteins are less readily precipitated by alcohol from their solutions after addition of acids or alkalies. Solutions of protein salts have also a greater viscosity than unionised protein. The same observation was made by Laqueur and Sackur(13) in case of casein. Fischer(14) has demonstrated the increased imbibition of water by gelatine, muscle and other protein bodies if acid or alkali be added to the solution in which the protein fragments are placed. How far this is due to the electrical charge borne by the protein particles in this condition is difficult to say. Chiari(15) has recently come to the conclusion, from experiments with gelatine, that the minimum imbibition of water takes place in fluids whose reaction (hydrogen-ion concentration equal to 2×10^{-5} normal) coincides with the iso-electric point of the protein.

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[1] See Table XXVI, p. 276. In Exp. 1, $K=1\cdot65$ is a misprint for $K=0\cdot65$.

The increased reaction-velocity of protein denaturation on addition of acids and alkalies falls into line with these observations. We may suppose the protein salt to be in more intimate association with the water which is known to be necessary for the denaturation-change.

The addition of neutral salts to acid or alkaline solutions of proteins has been shown to produce an analogous antagonistic effect in all the above instances. Precipitability by alcohol is restored, viscosity is lowered (Pauli and Handovsky and Schorr, *loc. cit.*), imbibition of water is reduced (Fischer, Chiari), and the reaction-velocity of denaturation in acid solution has also been shown by us to be greatly reduced in presence of neutral salts ((2) p. 17).

SUMMARY AND CONCLUSIONS.

1. Denaturation-rate of egg-albumen in alkaline solutions is shown to be increased with increasing concentration of hydroxyl-ions exactly as was previously shown to be the case with increase of hydrogen-ion concentration in acid solution.

2. Continuous removal of hydroxyl-ions as denaturation proceeds takes place in alkaline solution, just as in acid solution we found hydrogen-ion concentration to diminish.

3. If precautions are taken to keep the concentration of hydroxyl-ions constant during the process, denaturation proceeds as a reaction of the first order (as was also shown in the case of acid). If no such device is employed, denaturation-rate does not remain proportional to the concentration of unchanged albumen, but departs farther and farther from this value owing to the progressive fall in "alkalinity."

4. The influence of acids and alkalies on denaturation-rate is compared with their effect on the viscosity and precipitability by alcohol of protein solutions and upon the imbibition of water by protein; it is suggested that protein in the form of salts is in more intimate association with water.

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No. 4

ON THE "HEAT COAGULATION" OF PROTEINS. PART IV. THE CONDITIONS CONTROLLING THE AGGLUTINATION OF PROTEINS ALREADY ACTED UPON BY HOT WATER.

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Introduction. It has been a matter of common experience since the "heat coagulation" of proteins was first studied, that unless the reaction and salt-content of a protein solution be adjusted within certain limits no precipitate occurs on heating the solution.

Hardy (1) first pointed out that precipitation or coagulation of proteins under the influence of hot water involved two distinct processes, viz. (1) denaturation[1], and (2) subsequent agglutination of the denaturated particles. The same view was afterwards expressed by Pauli and Handovsky (2) (p. 425).

What precisely happens when denaturation of albumen in solution occurs, we are not yet able to say. In cases where agglutination is absent and the solution may remain completely limpid, its viscosity will be found to have considerably increased. This accession of viscosity in an emulsoid colloid indicates, as shown by Hatschek (3), that the volume of the dispersed phase has increased at the expense of the continuous phase, in other words the colloidal aggregates of protein have taken up more water. At the same time the conditions determining the dispersion of the colloid are altered, for as shown by Hardy (4), the protein can now be completely precipitated by adjusting the reaction so as to render the particles iso-electric with the solution. Small quantities of acid or alkali disperse them and they acquire the property of travelling in an electric field (Hardy (5)). If the amount of acid or alkali present does not exceed what is necessary to obtain a translucent liquid, particles can be seen under the ultra microscope. In the original albumen solution, before heating, the dispersion or solution of the protein *is not dependent upon electric charge* and Michaelis[1], whose important work will be referred to directly, concludes that the properties of the colloidal solution of albumen have by heating been changed from those of an "emulsoid" or hydrophilic colloid to those of a typical suspensoid. Dispersions of denaturated proteins certainly possess the character of suspensoids in so far that the suspension owes its existence to the electrical charge upon the particles, but their other properties, *e.g.* high viscosity and low surface tension, suggest that they are at the same time hydrophile and that they do not fall into either category but form an intermediate group with some of the properties of both.

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[1] We have adopted the term "denaturation" in conformity with the German equivalent "Denaturierung."

"Denaturated" egg- or serum-albumen when dispersed in weak alkali, is precipitated by dialysis, by saturating with sodium chloride, potassium chloride or magnesium sulphate (Starke (7)) and by ammonium sulphate in lower concentration than is necessary before heating (Moll (8)). That is to say, these denaturated proteins have acquired some of the characteristics peculiar to globulin. Indeed both Starke and Moll are of opinion that albumen is converted into globulin by heating. Michaelis and Rona ((9) and (10)) have shown that the state of colloidal suspension of serum globulin (eu-globulin) resembles that of heat-denaturated serum-albumen, but these resemblances are concerned with the conditions of dispersion only and do not imply chemical identity between these proteins. Since, according to Abderhalden ((11) and (12)), the crystalline serum-albumen of the horse affords no glycocol on hydrolysis whereas the globulin yields 3·5 % of this amino-acid, these two proteins would appear to possess a fundamentally different chemical constitution.

On the other hand, underlying the change in colloidal conditions produced by heating a solution of albumen, some chemical changes in the protein molecule have also been traced. Hopkins (13) found that the determination of sulphur in pure egg-albumen which had been coagulated by heat gave uncertain and low results compared with those made upon the original protein precipitated by alcohol, and in the case of crystalline serum-albumen Moll (*loc. cit.*) found that approximately one-third of the sulphur was split off by heat coagulation. The capacity of the protein to combine with acids is also increased on coagulation by hot water (Chick and Martin (14) and (15)). We have sought for evidence of hydrolytic cleavage by determining whether, after the action of hot water, there was any increase in the amount of titratable carboxyl groups as indicated by the formalin method of Sörensen (16). In the case of whole serum this was found to be the case, but with solutions of pure proteins (crystalline egg-albumen, and re-purified pseudoglobulin from horse-serum) we obtained no evidence of hydrolysis when the solutions were heated to 100° C. for two hours in presence of just sufficient alkali to prevent precipitation.

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[1] *Physik.-chem. d. Kolloide, Richter-Koranyi's Hdb.* II. p. 391; see also Michaelis and Mostynski (6).

INFLUENCE OF VARIOUS FACTORS UPON THE FINAL STATE OF AGGREGATION OF PROTEIN PARTICLES DENATURATED BY HEATING.

(1) *The influence of reaction.*

A slightly acid reaction is necessary in order to completely precipitate a solution of albumen by heating. The first step towards an interpretation of this fact was made by Hardy ((4) and (5)), who examined the electrical properties of a suspension of denaturated egg-white produced by heating a 1 in 10 dilution of this substance, and found that the protein was negatively charged in alkaline solutions and positively charged in acid solution. Further, as the iso-electric point was approached from either side, the stability of the hydrosol diminished, and at the iso-electric point was reduced to zero and precipitation ensued.

Michaelis (17) determined the direction in which unheated serum-albumen wandered in an electric field when the concentration of hydrogen-ions was varied. Different mixtures of primary and secondary phosphates were mixed with the albumen solution to obtain the variations in reaction and precautions were taken to obviate the secondary effects of electrolysis. Michaelis found that the iso-electric point for this protein was considerably on the acid side of neutral, and, in his earlier experiments, determined this point to correspond to a concentration of hydrogen-ions equal to 10^{-6} normal. From these results he concluded that the "relative acidity" of this protein was between 10^2 and 10^3.

Later Michaelis and Mostynski (6) investigated the iso-electric point of dialysed serum-albumen of the ox both in its natural condition and after denaturation by heat. The reaction of the solution was adjusted by different mixtures either of phosphates or of acetic acid and sodium acetate. Both with natural and denaturated serum-albumen the iso-electric point was found to lie at a concentration of hydrogen-ions ranging from 0·77 to 1×10^{-5} normal. They also determined the optimal acidity for agglutination of the denaturated serum-albumen by adding a small quantity of this to mixtures of acetic acid and sodium acetate in varying proportions. The quickest and most complete agglutination occurred in the mixture with a concentration of hydrogen-ions equal to $\cdot 82 \times 10^{-5}$, which corresponds with the observed value for the iso-electric point. Michaelis and Mostynski agree with Hardy in the interpretation of these results, namely, that at the iso-electric point the charge on the protein aggregates is minimal as they possess an equal tendency to dissociate as positive or negative ions and are not prevented from coming together under the influence of surface tension.

Michaelis and Rona (9) have re-determined the position for optimal flocculation of denaturated serum-albumen with greater accuracy, and have come to the conclusion that in the absence of other electrolytes this occurs at a concentration of hydrogen-ions equal to $\cdot 3 \times 10^{-5}$ normal, *i.e.* somewhat lower than the previously obtained value. The additional observations of Michaelis and Rona on the effect of neutral salts upon agglutination we shall have occasion to refer to later (p. 270). The optimal acidity for flocculation has also been recently studied by Sörensen and Jürgensen (18). They determined the influence of acidity of the solution upon the amount of protein which could be separated by subsequently boiling the solution. They added varying quantities of ·1 N hydrochloric, sulphuric and acetic acid to diluted horse-serum and egg-white, heated the solutions in a water bath at 100° C., filtered them and estimated the nitrogen in the filtrate. The latter was found to be minimal after addition of a certain definite amount of either acid. Excess of the stronger acids interfered with coagulation more readily than excess of acetic acid.

We have made a number of observations during the last three years, the results of which completely confirm the general conclusion arrived at by Michaelis and his collaborators and by Sörensen and Jürgensen. A few of our results are worth mentioning as the experiments were planned so as to ascertain the range of acidity within which a coagulable protein may be separated from a solution containing it.

Table I illustrates this in the case of diluted horse-serum to which varying amounts of acetic acid had been added. Column 3 shows the range (10–40 c.c. N/100 acid added) over which sufficient aggregation of the denaturated particles occurred to permit of complete separation of the protein by means of a Berkefeld filter. The 4th column shows the range (18–25 c.c. N/100 acid) over which the protein was kept back by filtering through paper.

TABLE I. *10 c.c. of a 43 % solution of horse-serum taken in each case and made up to a total volume of 80 c.c. by addition of water and 1/100 N acetic acid.*

Amount in c.c. of N/100 acetic acid (or equivalent) added	Appearance of the solution after heating 30 minutes in boiling water	Result of filtering the heated solution through	
		Berkefeld filter	Paper filter
0	Translucent	Filtrate contains protein *	—
5	Opalescent	,, ,, ,,	—
7·5	,,	,, ,, ,,	—
10	,,	Filtrate protein-free	98 % of total protein in filtrate.
12·5	Contains precipitate ...		12 % ,, ,, ,,
15	Clear solution, contains precipitate		9 % ,, ,, ,,
18 to 25	,, ,, ,,		Filtrate protein-free.
27	,, ,, ,,		9 % protein in filtrate.
30	Milky solution, contains precipitate		58 % ,, ,, ,,
40	,, ,, small precipitate		94 % ,, ,, ,,

* Also true after filtration through Doulton filter.

In Table II are set forth the results of a similar experiment with crystalline egg-albumen to which N/100 HCl had been added. Within a small range of acidity the particles aggregate sufficiently to be filtered off by means of filter paper; outside this range of optimum acidity, although not visible to the naked eye, they are for a considerable distance on both sides still sufficiently aggregated to be stopped by a Berkefeld filter. Beyond this second range the particles are too small to be kept back but a proportion of them are visible with the ultra-microscope.

In the experiment detailed in Table III the solution of egg-white was denaturated first and the reaction subsequently adjusted by the addition of N/100 acetic acid. Complete agglutination occurred over a considerable range, but took place more slowly at either end.

TABLE II. *Dialysed* solution of pure egg-albumen,* 1 %. *Total volume,* 10 *c.c.*

No. of exp.	Amount in c.c. of N/100 HCl (or equivalent) added in total volume of 10 c.c.	Appearance of solution after heating 15 mins. in boiling water	Result of filtering the heated solution through paper
1	0	Coagulum in a milky solution	Filtrate contains protein
2	0·5	Coagulum in a clear solution	Filtrate contains trace of protein ... ...
3	1·0†	Coagulum in a clear solution	Filtrate protein free
4	1·5	Opalescent, jelly-like	,, ,, ,,
5	2·0	Opalescent solution	,, ,, ,,
6	5·0	Clear solution ...	Filtrate contains all the protein ... ...

* If it contains any salt the range of agglutination at 100° C. is so much extended that an optimum acidity is difficult to detect.

† Hydrogen-ion concentration $= 10^{-4\cdot34}$ normal, determined electrically.

TABLE III. *Denaturated* 3 % *egg-white solution;* 5 *c.c. made up to a total volume of* 8 *c.c.*

Amount in c.c. of N/100 acetic acid added (or equivalent) in total volume of 8 c.c.	Appearance of solution after standing 24 hours	Result of filtering the solution through	
		paper	Berkefeld filter
0	No agglutination	—	Filtrate contains small amount of protein
1·3	,, ,,	—	—
1·4	,, ,,	—	—
1·5	Almost complete agglutination	Filtrate contains trace of protein	—
1·7	Complete agglutination	Filtrate protein-free	—
2·5			—
3			—
4			—
4·5			—
5	Partial agglutination	—	—
6	,, ,,	—	—
7	Trace of agglutination	—	—

The influence of the nature of the acid. The following experiments, made with diluted horse-serum and hydrochloric, acetic and butyric acids, show, as was to be expected from the observations of Hardy and Michaelis, that the weakest acid gives the widest range within which the denaturated protein can be completely separated by filtration through a paper filter. The results are given in Tables IV, V and VI, and graphically set forth in Fig. 1. After heating the various solutions to boiling point, the precipitates were caught on a weighed filter paper and weighed after washing and drying at 110° C.

All three acids have an equal effect in inducing agglutination of the protein particles up to the addition of 13–14 c.c. N/100 acid, at which point precipitation becomes complete and a protein-free filtrate is obtained. On further addition of acid, the effect of the three acids differs. With HCl the zone of complete precipitation extends only over a very small range (from *A* to *X*, Fig. 1), viz. up to the addition of 15·6 c.c. 1/100th normal HCl; beyond this the coherent precipitate becomes gradually less until in the solution containing 19·6 c.c. it is almost entirely dispersed. With acetic acid and butyric acid the range of

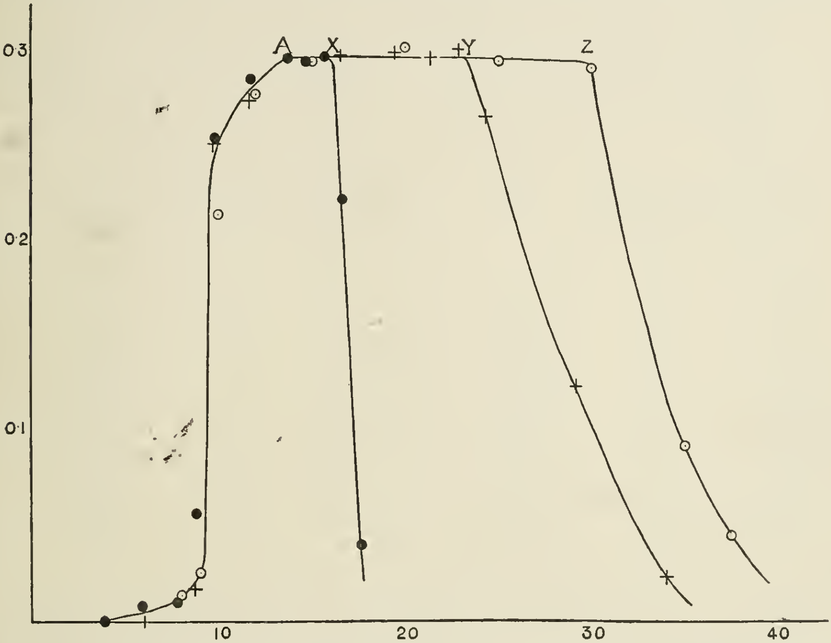

Fig. 1. Amount of protein precipitated which can be separated by a paper filter when 10 c.c. diluted horse serum (42·9 °/₀) are acidified with varying quantities of hydrochloric, acetic and butyric acids and heated for two hours at 100° C. (See Tables IV, V, and VI.) Total volume 80 c.c.

Ordinates = weight of coagulum in grams.
Abscissæ = no. of c.c. N/100 acid added.
——●—— = HCl.
——+—— = $H_2C_4O_2$.
——○—— = $H_4C_8O_2$.

complete agglutination continues until the addition of 22·8 c.c. and 30 c.c. 1/100 normal acid respectively (from *A* to *Y* and *Z* respectively, Fig. 1); on adding more acid the amount of the precipitate which can be filtered through paper diminishes, but more gradually than was the case with HCl.

TABLE IV. 10 *c.c. diluted horse-serum* (42·9 %) *acidified with varying quantities of hydrochloric acid and heated for two hours at* 100° *C. Total volume* = 80 *c.c.*

Amount of 1/100 N.HCl added in c.c.	Amount of H_2O added in c.c.	Amount of precipitate in grms.
0	70	0
1·95	68	0
3·9	66	0
5·9	64	·0078
8·8	61	·0100
9·8	60	·2561
11·7	58	·2873
13·7	56	·2988
14·7	55	·2969
15·65	54	·2996
16·6	53	·2238
17·6	52	·0407
19·6	50	small trace

TABLE V. 10 *c.c. diluted horse-serum* (42·9 %) *acidified with varying quantities of acetic acid and heated for two hours at* 100° *C. Total volume* = 80 *c.c.*

Amount of 1/100 N.$C_2H_4O_2$ added in c.c.	Amount of H_2O added in c.c.	Amount of precipitate in grms.
6	64	trace
7·8	62	·0082
8·7	61	·0172
9·7	60	·2529
11·65	58	·2759
14·55	55	·2943
16·5	53	·3003
19·4	50	·3020
21·35	48	·2994
22·8	46·5	·3041
24·3	45	·2684
29·1	40	·1256
34·0	35	·0237

TABLE VI. 10 *c.c. diluted horse-serum* (42·9 %) *acidified with varying quantities of butyric acid and heated for two hours at* 100° *C. Total volume* = 80 *c.c.*

Amount of 1/100 N.$C_4H_8O_2$ added in c.c.	Amount of H_2O added in c.c.	Amount of precipitate in grms.
8	62	·0140
9	61	·0254
10	60	·2158
12	58	·2796
15	55	·2972
20	50	·3052
25	45	·2980
30	40	·2946
35	35	·0936
37·5	32·5	·0459

These results show very clearly the increased working range which can be obtained by using a slightly ionised acid for acidification.

The results of two further experiments with acetic and butyric acids, in which the hydrogen-ion concentration of the various solutions was determined prior to boiling, are detailed in Tables VII and VIII. The observations made in the region of maximum precipitation were not sufficiently numerous to define the optimal hydrogen-ion concentration with exactitude, but the range over which maximal agglutination occurred was almost the same in both instances, *i.e.* ·47 to $1·35 \times 10^{-5}$ normal and ·6 to $1·74 \times 10^{-5}$ normal when the solutions were acidified with acetic and butyric acids respectively. The mean hydrogen-ion

TABLE VII. 10 *c.c. diluted horse serum** (42·9 $^0/_0$) *heated for* 30 *mins. at* 100° *C. after addition of varying amounts of acetic acid. Total volume* = 80 *c.c.*

Amount of N/100 $C_2H_4O_2$ in c.c.	Amount of H_2O added in c.c.	Amount of the precipitate obtained in grms.	Concentration of H+ in solutions before heating, in terms of normality
0	70	0	$10^{-7.8}$ normal ($0·16 \times 10^{-7}$ normal)
5	65	0	$10^{-7.40}$,, (0·4 ,, ,,)
7·5	62·5	0	$10^{-7.27}$,, (0·54 ,, ,,)
10	60	·0074	$10^{-6.86}$,, (1·3 ,, ,,)
12·5	57·5	·2518	$10^{-6.20}$,, (6·4 ,, ,,)
15	55	·2614	$10^{-5.78}$,, (17 ,, ,,)
18	52	·2874	$10^{-5.33}$,, (47 ,, ,,)
22	48	·2912	$10^{-5.05}$,, (89 ,, ,,)
25	45		$10^{-4.87}$,, (135 ,, ,,)
27	43	·2629	$10^{-4.82}$,, (152 ,, ,,)
30	40	·1187	$10^{-4.78}$,, (166 ,, ,,)
40	30	·0160	$10^{-4.49}$,, (324 ,, ,,)

* A different sample from that in Tables IV–VI.

TABLE VIII. 10 *c.c. diluted horse-serum** (42·9 $^0/_0$) *heated for* 30 *mins. at* 100° *C. after addition of varying amounts of butyric acid. Total volume* = 80 *c.c.*

Amount of N/100 $C_4H_8O_2$ in c.c.	Amount of H_2O added in c.c.	Amount of the precipitate obtained in grms.	Concentration of H+ in solutions before heating, in terms of normality
0	70	0	$10^{-7.8}$ normal ($0·16 \times 10^{-7}$ normal)
10	60	·0018	$10^{-6.85}$,, (1·4 ,, ,,)
14	56	·2563	$10^{-5.96}$,, (11·0 ,, ,,)
20	50	·2874	$10^{-5.22}$,, (60 ,, ,,)
27	43	·2906	
29	41	·2872	
30	40	·2768	$10^{-4.76}$,, (174 ,, ,,)
40	30	·0448	$10^{-4.63}$,, (229 ,, ,,)
60	10	·0	$10^{-4.33}$,, (468 ,, ,,)

* A different sample from that in Tables IV–VI.

concentration of this range, about 10^{-5} normal, does not differ greatly from that observed by Michaelis and his collaborators for serum-albumen.

The above results support the conclusion of Hardy and Michaelis that the facility with which particles of denaturated protein can be aggregated is conditioned by the concentration of hydrogen-ions in this solution. By working at the high temperature of our experiments, 98° C. to 100° C., the range throughout which agglutination takes place is more extensive than under the conditions of Michaelis' observations.

An explanation of the fact that the iso-electric point for proteins is on the acid side of neutral may be found in the amphoteric nature of the protein molecule. Michaelis (17) considered the position of the iso-electric point to depend upon the relative tendency of the protein to dissociate as acid and base respectively. From theoretical reasoning he concludes that the ratio of hydrogen- to hydroxyl-ions (" relative acidity ") at the iso-electric point, is equal to the ratio of the dissociation constants of the protein as acid and base respectively. This was later demonstrated by Michaelis and Davidsohn (19) in the case of amino-benzoic acid where these constants have been experimentally determined. On this view, when sufficient acid is present to reduce ionisation of the carboxyl-group to the same degree as that of the amino-group, the protein becomes iso-electric and aggregates owing to surface tension.

The aggregation of protein particles by electrolytes which will next be discussed seems, however, to be a phenomenon of a different order.

(2) *The influence of neutral salts.*

Pauli and Handovsky (2) studied the influence of a large series of neutral salts upon the coagulation temperature of proteins and (p. 425) attributed the effect of salts in raising the " coagulation temperature " of dialysed ox-serum entirely to their hindering the agglutination of the particles of the denaturated albumen. That this exclusion of any effect of salts upon denaturation-rate is unjustified follows from our own observations ((15), p. 17). Michaelis and Rona (9) found that if serum-albumen were denaturated by heating in alkaline solution, agglutination of the particles took place more readily on addition of acid, if the material had been previously dialysed. The hindering effect of salts was noticeable in the case of NaCl when it was present to the extent of ·06 %, and the process was altogether prevented when the solution contained more than 0·6 %. In the case of the denaturated proteins of egg-white, on the other hand, agglutination has been shown to be markedly assisted by the presence of neutral salts in small quantities (Hardy (5)).

We have found this to be true also of serum proteins *if the salt is added before heating.* In the present communication we are, however, only concerned with the agglutination of previously denaturated proteins.

The apparent difference in *character* between the influence of neutral salts on the denaturated proteins of serum and egg-white respectively is well seen from the results of experiments (*a*) and (*b*) below.

(*a*) *Horse-serum.*

The solution of denaturated horse-serum was prepared as follows: 10 c.c. diluted serum (43 %), 30 c.c. water and 8 c.c. 1/100th normal acetic acid, the addition of which raised the concentration of hydrogen-ions in the solution to about 10^{-6} normal, were heated for half-an-hour on a water bath at 100° C. A milky solution was obtained which contained no visible particles. This solution was dialysed for two days against tap-water and then diluted with distilled water, until its protein-content was the same as that of the previous experiments (see Tables IV to VIII). This material agglutinated readily in the cold on addition of a suitable amount of acid.

The effect of salts upon agglutination was then studied by comparing velocity of agglutination in presence of a series of different concentrations of acetic acid, both in absence of salts and after addition of various small amounts of sodium chloride and ammonium sulphate.

Agglutination-rate was measured by the time which elapsed after adding the salt to the various solutions before particles appeared which were distinctly visible to the naked eye. The reciprocal of this time, in minutes, was taken as an index of the agglutination-rate. The experiments were made in a thermostat at 37° C. in order that the rate of agglutination might be convenient for study.

The results of four experiments with ·01, ·10, ·2 and ·5 % NaCl respectively are given in Table IX, where column 1 contains the results of a control experiment in which no salt was added, for comparison.

It is seen that concentrations of sodium chloride up to ·1 % are without much effect, but beyond this the rate of agglutination is progressively lowered by the addition of salt and the range of acidity over which agglutination can take place is also curtailed (see Table IX, experiments with ·2 and ·5 % NaCl). The same results are graphically shown in the five curves of Fig. 2, where agglutination-rate is plotted against the amount of acetic acid added. These curves also show very well the effect of salt in necessitating the addition of more acid[1] before the point of optimum acidity for agglutination is reached, or, in other words, in inducing agglutination in solutions otherwise too acid for precipitation.

[1] See also Sörensen and Jürgensen, *loc. cit.* (18), p. 419.

TABLE IX. 5 *c.c. of a solution containing* 5·3 % *of horse-serum, dialysed, diluted to* 8 *c.c. by addition of water, salt and acid. Temp.* = 37° *C.*

Amount of N/100 acetic acid (or equivalent) added in c.c.	Rate of agglutination, as measured by the reciprocal of *t*, the time, in minutes, elapsing after mixing before particles were distinctly visible in the solution, when the concentration of NaCl was				
	0	·01 % (·0017 normal)	·10 % (·017 normal)	·20 % (·034 normal)	·50 % ·085 normal
	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$
0·7	0	0	—	—	—
0·8	2·7	1·7	< 0·03	—	—
0·9	—	3·0	0·92	—	—
1·0	—	—	2·63	—	—
1·1	—	—	—	—	—
1·2	2·9	—	3·33	—	—
1·3	—	3·0	3·57	0·03	—
1·4	—	—	3·85	—	—
1·5	3·0	3·3	2·38	0·5	—
1·6	1·09	3·0	1·49	—	—
1·7	0·04	0·1	0·92	0·85	—
1·8	0	—	0·50	—	·07
1·9	—	—	0·15	1·49	·05
2·0	—	—	—	—	·04
2·1	—	—	—	1·72	·03
2·2	—	—	—	—	·02
2·3	—	—	—	1·09	—
2·4	—	—	—	—	< ·017
2·5	—	—	—	0·60	—

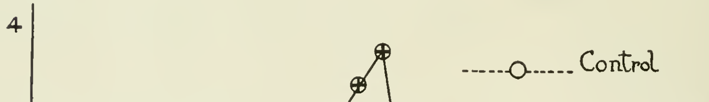

Fig. 2. Agglutination of denaturated horse serum (5·3 %) dialysed, and after addition of NaCl to various concentrations, at 37° C. (See Table IX.)

Ordinates = agglutination-rate, as measured by the reciprocal of the time, in minutes, elapsing before the appearance of visible particles.

Abscissæ = c.c. of N/100 acetic acid (or equivalent) in total volume of 8 c.c.

In Table X and Fig. 3 are given the results of similar experiments, showing effect of ammonium sulphate in concentrations of ·1 and ·5 $^0/_0$ respectively. The action of the salt is the same as that of sodium chloride except that the hindering effect upon agglutination is stronger, being already very well marked in a concentration of ·1 $^0/_0$. The alteration of the best agglutinating conditions to those of solutions containing a higher concentration of acid is also more marked in this instance.

TABLE X. *5 c.c. of solution containing 5·3 $^0/_0$ horse-serum, dialysed, diluted to 8 c.c. by addition of water, salt and acid. Temp. = 37° C.*

Amount of N/100 acetic acid (or equivalent) added in c.c.	Rate of agglutination, as measured by the reciprocal of t, the time, in minutes, elapsing after mixing before particles were visible, when the concentration of Am$_2$SO$_4$ was		
	0	·1 $^0/_0$ (·015 normal)	·5 $^0/_0$ (·076 normal)
	$\frac{1}{t}$	$\frac{1}{t}$	$\frac{1}{t}$
0·6	1·09	—	—
0·7	5·88	<0·01	—
0·9	—	0·47	—
1·0	—	1·5	—
1·1	—	2·0	—
1·2	5·88	—	—
1·3	4·00	3·6	—
1·4	1·33	—	—
1·5	0·125	3·0	—
1·7	—	3·0	—
1·9	—	2·0	—
2·0	—	—	·003
2·1	—	1·5	—
2·2	—	—	·003
2·3	—	0·9	—
2·5	—	0·75	·005
2·8	—	0·33	<·003
3·0	—	—	—
3·2	—	0·10	—

In the cases where agglutination was slow in beginning it was usually also incomplete; the solutions containing ·5 $^0/_0$ sodium chloride or ammonium sulphate never agglutinated completely during the time they were under observation.

(b) *Egg-white.*

A milky solution of denaturated egg-white was employed, similar to that used in investigating the effect of acid upon agglutination (see above, p. 265). 5 c.c. denaturated diluted (1 in 32) egg-white was further diluted to 8 c.c. by addition of various amounts of acid, salt solution and water, so that the solutions contained about ·2 $^0/_0$ protein.

A series of solutions were thus prepared with various amounts of acetic acid, and similar series were made up containing in addition 1 and 2 % NaCl as well as the salts naturally occurring in the solution.

Precipitation occurred slowly and completeness or otherwise of the agglutination was observed in the different mixtures after they had stood for 24 hours at room temperature.

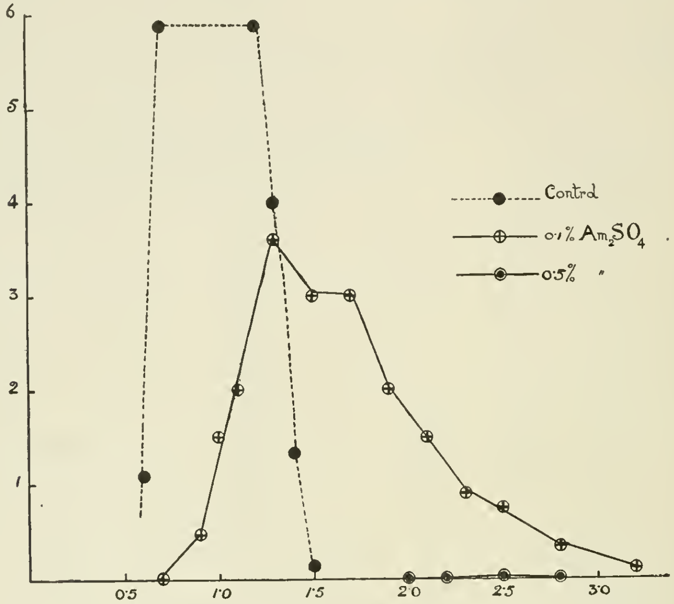

Fig. 3. Agglutination of denaturated horse serum (5·3 %) dialysed, and after addition of Am_2SO_4 to various concentrations, at 37° C. (See Table X.)

Ordinates = agglutination-rate, as measured by the reciprocal of the time, in minutes, elapsing before the appearance of visible particles.

Abscissæ = c.c. of N/100 acetic acid (or equivalent) in a total volume of 8 c.c.

The addition of salt was found to assist agglutination and to very greatly extend, on the acid side, the zone of acidity over which it was complete. In the absence of salt, it was found that complete agglutination took place only in concentration of acetic acid lying between 1·5 c.c. and 5·0 c.c. N/100 acetic acid (or equivalent) in a total volume of 8 c.c. In presence of 1 % NaCl the zone of agglutination extended up to an addition of 3 c.c. N/10 acetic acid, and in the presence of 2 % NaCl up to an addition of 16 c.c. N/10 acetic acid (or equivalent) in the same total volume.

The addition of salts also greatly assists the agglutination of pure crystalline egg-albumen. A dialysed solution of crystals, containing 1 % protein, will only precipitate completely on boiling if the reaction is carefully adjusted (see Table II above). A 1 % solution of undialysed crystals on the other hand prepared by the method of Hopkins and Pinkus(20) and containing ·3 to ·4 % Am_2SO_4 will coagulate perfectly on boiling over a wide range of acidity (hydrogen-ion concentration $10^{-3·1}$ normal to $10^{-7·4}$ normal, see our previous papers, (14), p. 423 and (15), p. 5).

The effect of ammonium sulphate was also well shown in the following experiment.

A dialysed solution of crystals in its natural reaction on dialysis (hydrogen-ion concentration equal to 10^{-5} normal) was diluted to form a series of solutions containing 1 % protein with various small concentrations of ammonium sulphate; these were then heated for 15 mins. on a water bath at 100° C. In the absence of salt a milky solution was obtained containing a partial coagulum.

Agglutination was rendered almost complete by ·01 % ammonium sulphate. In presence of ·1 % ammonium sulphate complete precipitation took place and the filtrate after filtration through ordinary filter paper was protein-free.

The apparently opposite effect of salts upon the agglutination of egg- and serum-proteins respectively might conceivably have been due to some effect of salts in lowering surface tension at the interface of the colloidal particles and the liquid in the case of serum-protein, and raising it in the case of egg-protein. We endeavoured to test this by seeing whether in the former instance we could obtain evidence of adsorption of salts by the colloid; we found no indication of adsorption in either case.

It seemed likely, therefore, that some light would be thrown upon the different effect of salts upon the agglutination of serum- and egg-proteins by a comparative study of a wider selection of electrolytes and the radicles of which they are composed. Hardy(5) pointed out that a solution of denaturated egg-white offered a satisfactory analogy with certain inorganic colloidal solutions upon which the precipitating action of electrolytes had been more completely investigated. He showed that in acid solutions the protein particles carried a positive electric charge and were sensitive to the anion of the electrolyte added, precipitating power of which was determined by its valency. Similarly in alkaline solutions, where the protein particles are negatively electrified, they display great sensitiveness to the potency of the kation.

The experiments in Table XI are in confirmation of the above.

Diluted egg-white was filtered, heated to about 95° C. on a water bath, precipitated by acidification, filtered, washed free of salts and puddled up with water to make a fine suspension which was at or near the iso-electric point. 5 c.c. of this protein suspension was diluted to 10 c.c. by addition of acid or alkali, salt solution etc., and the effect of electrolytes on agglutination was studied in the case of sodium and barium chlorides and sodium sulphate.

TABLE XI. 5 *c.c. of a suspension of denaturated diluted egg-white, at or near the iso-electric point, diluted to* 10 *c.c. with addition of acid or alkali, salt solution, etc.*

+ + + = complete agglutination.
+ + = almost complete agglutination.
+ = partial agglutination.
+ − = almost complete dispersion.
− = complete dispersion, opalescent solution.

Salt added	Concentration, in terms of normality	No. of c.c. N/100 HCl (or equivalent) added in total of 10 c.c.					No addition of either acid or alkali	No. of c.c. N/100 NaOH (or equivalent) added in total of 10 c.c.	
		10	2	1	0·5	0·25		0·5	1·0
Control	...	−	+ −	+	+	+ +	+ + +	−	−
NaCl	·05	...	+	+	+ + +	...	+ + +	+	−
...	·10	+	+ +	+ + +	+ + +	...	+ + +	+	−
Na_2SO_4	·05	+ +	+ + +	+ + +	...	...	+ + +	+	−
$BaCl_2$	·05	+	+	+ +	...	...	+ + +	+ + +	+ + +

The general impression given is that salts assist coagulation. In the case of sodium salts, while the sulphate is much more effective than the chloride on the acid side, both salts exert only a slight effect upon the alkaline side which is equal in the two cases. When the action of the two chlorides is compared it is evident that the action of the barium salt greatly exceeds that of the sodium salt in causing precipitation in the alkaline solution.

Similar experiments with denaturated serum-proteins are given in Table XII. The material consisted of dialysed horse-serum which after diluting and heating was still on the alkaline side of the iso-electric point. In low concentration the effect of salts is exactly analogous to that in the case of egg-proteins, but in concentrations greater than ·01 normal, *all* the salts hindered agglutination.

This difference in the action of electrolytes upon denaturated egg- and serum-proteins disappears when a trivalent anion, such as a citrate, is used. Sodium citrate in low concentration agglutinates particles of both proteins when they are dissolved in acid, and on further addition of the salt both are again dispersed. The salt was prepared so as to be absolutely neutral by titrating citric acid with standard sodium hydroxide, as otherwise the effect is complicated by alteration of the reaction of the solution.

For the experiment set out in Table XIII the material used was a dilute solution of egg-white, boiled, dialysed and further diluted until its protein-content was equal to ·1 %. It was then rendered slightly acid so that the protein particles were dispersed. On addition of sodium

TABLE XII. *Dialysed horse-serum, diluted 1 in 10, heated to 90°—100° C.; 5 c.c. of the milky fluid diluted to 10 c.c. with acid or alkali, salt solution and water.*

+ + + = complete agglutination.
+ + = almost complete agglutination.
+ = partial agglutination.
+ − = almost complete dispersion.
− = complete dispersion.

Exp.	Salt added	Concentration in terms of normality	No. of c.c. N/100 HCl (or equivalent) added in total of 10 c.c.									*	No. of c.c. N/100 NaOH (or equivalent) added in total of 10 c.c.				
			10	5·0	3·0	2·0	1·5	1·0	0·75	0·5	0·25		1·0	2·0	3·0	5·0	10
1	Control	...	+	+	+	+	...	+	+ + +	+ + +	+	+	+	+	+	+	−
2	NaCl	·01	..	...	...	...	+	+ + +	+ + +	+	...	...	...	...	...	...	−
3	,,	·05	...	..	+	+	...	+	..	+	...	...	...	...	...	...	...
4	Na_2SO_4	·005	...	...	...	...	+	+ + +	+ + +	+	...	...	...	...	...	...	...
5	,,	01	....	...	...	...	+ +	+ + +	+ +	+	...	...	...	...	...	...	...
6	,,	·05	...	+	+	+	...	+	...	...	...	...	...	...	...	...	+ −
7	,,	·1	...	...	+	+	...	+	...	...	...	...	...	...	...	...	...
8	$BaCl_2$	·005	...	...	...	+	...	+ +	+ + +	+ + +	...	+	+†	+†	...	...	...
9	,,	·01	...	...	...	...	...	...	+ +	+ +	...	...	...	...	...	...	...

* No addition of either acid or alkali.

† Rather better agglutination than in corresponding tubes without salt.

citrate, agglutination took place when the concentration of the salt was equal to ·0006 normal, but on further addition of the salt, at a concentration between ·002 and ·004 normal, the protein particles were again dispersed and, as was subsequently discovered, bore an electric charge opposite in sign to that originally carried. The concentrations of sodium chloride and sodium sulphate necessary for agglutination was ·10 and ·006 normal respectively. No upper limit for dispersion could be discovered in either case; agglutination remained perfect up to a concentration where precipitation (salting out) of the protein began.

TABLE XIII. *A colloidal solution of denaturated egg-white containing* 0·114 % *protein was obtained by dispersing the dialysed material by a minimal quantity of* (*a*) *acid and* (*b*) *alkali;* 5 *c.c. of the milky solution diluted with water and salt solution to* 10 *c.c.*

+ = complete agglutination. – = dispersion.

Concentration of protein %	Salt added	Concentration of salt in terms of normality	Agglutination	Concentration of salt, in terms of normality, necessary to cause	
				Agglutination	Dispersion
(*a*) *Original material dispersed by acid.*					
·057	NaCl	·06	–	·10	...
		·08	–		
		·10	+		
		·20	+		
		·50	+		
·057	Na_2SO_4	·004	–	·006	...
		·006	+		
		·01	+		
		·03	+		
		·05	+		
		·5	+		
·057	Na_3Cit (neutral)	*·0002	–	·0006	·004
		·0004	–		
		·0006	+		
		·0008	+		
		·001	+		
		·002	+		
		·004	–		
		†·006	–		
(*b*) *Original material dispersed by alkali.*					
·057	NaCl	·01	–	...	...
		·5	–		
		1·0	–		
		2·5	–		
(*b*) *Original material dispersed by alkali* (*cont.*).					
·057	$CaCl_2$	·01	–	·02	·5
		·02	+		
		·05	+		
		·1	+		
		·2	+		
		·5	–		
		1·0	–		
·057	$La(NO_3)_3$	·0001	–	·0002	...
		·0002	+		
		·001	+		
		·01	+		
		·05	+		
·011	$CaCl_2$	·001	–	·03	·5
		·01	–		
		·03	+		
		·05	+		
		·1	+		
		·2	+		
		·5	–		
		1·0	–		
·011	$La(NO_3)_3$	·00001	–	·00005	...
		·00005	+		
		·0001	+		
		·001	+		
		·005	+		
		·05	+		
		·1	+		
		·5	+		

* Particles positively charged, H^+ concentration $= 10^{-3·59}$ normal (2580×10^{-7} normal).

† Particles negatively charged, H^+ concentration $= 10^{-6·21}$ normal ($6·10 \times 10^{-7}$ normal).

A similar phenomenon was noticed when salts containing multivalent kations were added to alkaline solutions. A colloidal solution of denaturated egg-white in alkaline solution was agglutinated by calcium chloride in concentration of ·02 normal; dispersion, however, again set in at ·5 normal. Although lanthanum nitrate proved a powerful agglutinant, as would be expected from a salt of a trivalent metal, it did not cause dispersion in the concentration employed (see Table XIII).

TABLE XIV. *Horse-serum dialysed.* (*a*) *Heated in solution containing* ·17 *grm. protein and* 1 *c.c. N*/100 *NaOH per* 100 *c.c.* (*b*) *Heated in solution containing* ·056 *grm. protein and* 7·7 *c.c. N*/100 *HCl per* 100 *c.c.* 2·5 *c.c. of the above solutions diluted to* 5 *c.c. by addition of water and salt solution.*

	Concentration of protein %	Salt added	Concentration of salt in terms of normality	Agglutination[1]	Concentration of salt, in terms of normality, necessary to cause		Sign of electric charge of dispersed particles
					Agglutination	Dispersion	
(*a*)	·085	La $(NO_3)_3$	·00	–	·00006	·002	
			·00005	–			
			·00006	+ +			
			·0001	+ +			
			·0005	+ +			
			·0008	+ +			
			·001	+			
			·002	–			
			·005	–			+
	·085	$CaCl_2$	·005	–	·008	·02	
			·008	+			
			·01	+ +			...
			·02	–			
			·03	–			
	·0085	$CaCl_2$	·00	+ +	...	·02	–
			·005	+ +			
			·01	+ +			
			·015	+ +			
			·02	–			0
			·025	–			0
			·03	–			0
(*b*)	·028	Na_3Cit	·0001	–	·0008	·01	+
			·0005	–			+
			·0008	+ +			
			·001	+ +			
			·005	+			
			·01	–			
			·015	–			–
	·028	Na_2SO_4	·001	–	·01	·05	+
			·005	–			+ (slight)
			·01	+ +			
			·05	–			–

[1] + + = agglutination. + = partial agglutination. – = dispersion.

Some comparative experiments on the dispersing power of different salts upon denaturated serum-protein and its dependence upon valency are given in Table XIV. In the case of an acid dispersion the protein (·028 %), was first thrown down, and again dispersed by Na_2SO_4 in concentration of ·01 and ·05 normal respectively. In the case of sodium citrate the corresponding strengths were reduced to ·0008 and ·01 normal. A similar effect was shown by salts containing divalent and trivalent kations upon alkaline dispersions of the same protein, which was dissolved by $CaCl_2$ and $La(NO_3)_3$ in the low concentrations of ·02 and ·002 normal respectively.

The difference in effect of electrolytes upon denaturated serum- and egg-proteins both in acid and alkaline solutions is therefore one of degree only. For the agglutination of both there is an optimum concentration of electrolytes which diminishes as valency rises. Any excess beyond this optimum hinders agglutination and in some cases again disperses the protein. In the case of denaturated serum-proteins the particles are easily dispersed by excess of electrolytes but with egg-albumen this occurred only under the influence of multivalent ions. Hopkins and Savory(21) were evidently dealing with a similar set of phenomena in their work on Bence-Jones protein. They attributed the instability of the heat-coagulum to an extreme sensitiveness to the presence of electrolytes, the "solvent" action of which was especially marked when they consisted of multivalent ions.

Relation of the influence of salts upon agglutination to their effect upon hydrogen-ion concentration. Hardy(22) and Pauli and Handovsky(23) arrived at the conclusion that the hydrogen-ion concentration of acid solutions of protein was di~~minish~~ed by the addition of neutral salts. This conclusion was based on experiments made with indicators, a method which has since been proved to be untrustworthy in the presence of proteins and salts (Michaelis and Rona(24), Sörensen(25)). In our previous papers (14) and (15) we have described experiments confirming the observations of these authors, but showing that when acidity is determined electrically, salts reduced the hydrogen-ion concentration of protein solutions containing a small amount of acid. Further data we have obtained have shown the degree to which this occurs to be dependent upon the valency of the anion.

ed /

We have since discovered that there is a parallel action of electrolytes in reducing hydroxyl-ions in alkaline solutions. The experiments were made in solutions containing approximately 1 % pure egg-albumen crystals, dialysed. The salts employed were NaCl, Na_2SO_4 and $CaCl_2$, in concentrations varying from ·04 to ·13 normal. The results are given in Table XV below and show that the effect is to be attributed mainly to the kation of the salt and to be much increased with rising valency.

TABLE XV. *Reduction of hydroxyl-ion concentration on addition of neutral salts to alkaline protein-containing solutions.*

1 °/₀ solution, pure crystalline egg-albumen.

Salt added	Concentration of salt, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Calculated * concentration of hydroxyl-ions, in terms of normality
0	0	$10^{-8.88}$ normal = (·013 × 10^{-7} normal)	$10^{-5.25}$ normal (56 × 10^{-7} normal)
KCl	·05	$10^{-8.42}$ normal = (·038 × 10^{-7} normal)	$10^{-5.72}$ normal (19 × 10^{-7} normal)
K_2SO_4	·13	$10^{-8.19}$ normal = (·065 × 10^{-7} normal)	$10^{-5.95}$ normal (11 × 10^{-7} normal)
$CaCl_2$	·05	$10^{-7.87}$ normal = (·135 × 10^{-7} normal)	$10^{-6.27}$ normal (5·3 × 10^{-7} normal)

* Dissociation constant of water being taken as $10^{-14.14}$ (see Sörensen(25), p. 161).

The alteration of the reaction of protein-containing solutions by neutral salts is possibly brought about in two ways. The same phenomenon occurs[1] in the case of an amino-acid such as glycocol, where the addition of salt reduces the acidity of a solution containing dilute hydrochloric acid. In this case there is chemical combination between the amino-acid and the salt and definite crystalline compounds have been prepared. Reasoning from analogy, some part of the action described above may therefore be due to decreased hydrolysis of the protein-acid salt or protein-alkali salt, as the case may be, under the influence of a similar chemical union between the protein and the electrolyte. The enhanced effect of salts with di- and trivalent ions is, however, not explained on such an hypothesis.

From analogy with the observations of Whitney and Ober(26), which are referred to on p. 284 below, we are inclined to regard the effect of salts upon hydrogen-ion concentration as the result of selective adsorption of ions by the colloid, the attraction being for that ion bearing a charge of opposite sign. For an adequate explanation of the whole process it would be necessary first of all to possess some knowledge of the cause of the charge originally carried by the colloidal particles. This is still obscure in the case of inorganic colloids, and although the amphoteric nature of the molecule offers a plausible explanation of the initial charge carried by the protein, it is of no help in explaining the influence of electrolytes upon either the reaction of the solution or the agglutination of the particles.

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[1] Our colleague Dr Atkin kindly determined the hydrogen-ion concentration of solutions of glycocol and small quantities of HCl with and without the addition of NaCl.

The question arises as to how far the action of electrolytes in favouring or hindering agglutination, which has been described in the preceding section, may be explained by their effect on reaction. It is evident that a solution, the hydrogen-ion concentration of which is slightly greater or slightly less than that requisite for complete precipitation of the denaturated protein present, may, by the addition of a neutral salt, become adjusted to the degree of acidity appropriate for agglutination. Accordingly a set of experiments similar to those already described above were carried out, with solutions of denaturated egg- and serum- proteins, and the hydrogen-ion concentration was directly measured both before and after addition of the various salts.

The hydrogen-ion determinations were made as described in our previous paper (14) except that in the later experiments we abandoned the convenient device of connecting the two hydrogen cells by means of tubes plugged with filter papers and substituted capillary tubes of 1 mm. bore instead. Mr W. B. Hardy suggested to us that such a method, when dealing with charged colloidal particles, was open to objection, and a series of experiments made to investigate the matter showed that in the case of protein solutions a slight error was introduced. We are, however, satisfied that the magnitude of any error due to this practice is not such as to materially affect our previously published results.

Egg-white. The results of a set of experiments with denaturated egg-white are set out in Table XVI. The material was exactly similar to that employed above (Table XI) and was at or near the iso-electric point. In the absence of salts the agglutination-zone was found to be at a concentration of hydrogen-ions between 3 and 900×10^{-7} normal. Addition of Na_2SO_4 to a concentration of ·005 and ·05 normal extended the range on the acid side to a hydrogen-ion concentration of 1400 and $20{,}000 \times 10^{-7}$ normal respectively. At the same time the presence of the salt greatly reduced the hydrogen-ion concentration from what it would have been in its absence. Similarly, addition of barium chloride to a concentration of ·005 normal lowered the concentration of hydroxyl-ions and increased the range of agglutination on the alkaline side of the iso-electric point.

Horse-serum. The action of sodium sulphate in reducing hydrogen-ion concentration in solutions containing particles of denaturated serum-protein is similar to what occurs in the case of egg-proteins and, when

TABLE XVI. *A solution of diluted egg-white, filtered, heated, precipitated by addition of acid and washed free of salts, puddled up with water to make a fine suspension; 5 c.c. of the suspension diluted to 10 c.c. by the addition of acid or alkali, salt solution, water.*

Exp.	Salt added	Concentration of salt, in terms of normality	No. of c.c. N/100 HCl (or equivalent) added in total of 10 c.c.	No. of c.c. N/100 NaOH (or equivalent) added in total of 10 c.c.	Hydrogen-ion concentration, in terms of normality	Degree of agglutination
1	—	—	—	·5	$10^{-7.39}$ normal (0·405 × 10^{-7} normal)	Dispersed.
2	—	—	—	·25	$10^{-6.75}$ normal (1·77 × 10^{-7} normal)	,,
3	—	—	—	·17	$10^{-6.53}$ normal (3·0 × 10^{-7} normal)	,,
4	—	—	0·00	·00	$10^{-5.54}$ normal (28·9 × 10^{-7} normal)	Agglutinated.
5	—	—	0·08	—	$10^{-4.03}$ normal (934 × 10^{-7} normal)	Almost completely agglutinated.
6	—	—	0·17	—	$10^{-3.89}$ normal (1280 × 10^{-7} normal)	Slightly agglutinated.
7	—	—	0·25	—	$10^{-3.69}$ normal (2033 × 10^{-7} normal)	Dispersed.
8	—	—	0·50	—	$10^{-3.28}$ normal (5190 × 10^{-7} normal)	,,
9	—	—	1·0	—	$10^{-3.19}$ normal (6460 × 10^{-7} normal)	,,
10	Na_2SO_4	·005	0·5	—	$10^{-4.13}$ normal (736 × 10^{-7} normal)	Agglutinated.
11	,,	,,	0·75	—	$10^{-3.85}$ normal (1407 × 10^{-7} normal)	Almost completely agglutinated.
12	,,	,,	1·0	—	—	Almost completely dispersed.
13	,,	·05	—	·17	$10^{-6.78}$ normal (1·6 × 10^{-7} normal)	Almost completely agglutinated.
14	,,	,,	—	·25	$10^{-6.47}$ normal (3·4 × 10^{-7} normal)	,, ,, ,,
15	,,	,,	1·0	—	$10^{-4.00}$ normal (1000 × 10^{-7} normal)	Agglutinated.
16	,,	,,	5·0	—	$10^{-2.70}$ normal (20,000 × 10^{-7} normal)	,,
17	$BaCl_2$	·005	—	·5	$10^{-7.064}$ normal (0·86 × 10^{-7} normal)	,,

present in low concentration, agglutination may be assisted in acid solution, see Exp. 8, Table XVII. On the other hand, when this salt is present to the extent of ·05 normal, agglutination is prevented *even when the hydrogen-ion concentration may be the optimum for precipitation*, see Exp. 11, Table XVII.

Whatever may be the nature of the process involved in the phenomenon, it is clear that the change in reaction of protein solutions caused by electrolytes is inadequate to explain the influence of the latter upon agglutination.

The interpretation of the action of electrolytes in agglutinating and dispersing protein particles. At first sight it appears puzzling that a colloidal solution of denaturated protein should be destroyed by a certain concentration of an electrolyte and that solution should again take place on further addition of the same salt. Precipitation of inorganic colloidal solutions by electrolytes is apparently due to their discharge following the adsorption on the particles of that ion of the electrolyte which bears a charge opposite in sign to their own. Linder and Picton (27) found that when the negatively charged particles of arsenic trisulphide were thrown out of their colloidal solution by the addition of barium chloride, barium was present in the sulphide precipitate. Whitney and Ober (26) confirmed the observation of Linder and Picton and showed that an exactly

TABLE XVII. *Dialysed horse-serum, diluted* 1 *in* 10, *heated to about* 95° *C.; 5 c.c. milky fluid diluted to* 10 *c.c. by addition of acid or alkali, water, salt solution.*

Exp.	Nature of salt added	Concentration of salt, in terms of normality	C.c. N/100 HCl (or equivalent) added in total vol. of 10 c.c.	Hydrogen-ion concentration, in terms of normality	Degree of agglutination
1	—	—	0	$10^{-7.08}$ normal ($\cdot 83 \times 10^{-7}$ normal)	Dispersed.
2	—	—	0·5	$10^{-6.36}$ normal ($4 \cdot 35 \times 10^{-7}$ normal)	,,
3	—	—	0·6	$10^{-6.08}$ normal ($8 \cdot 22 \times 10^{-7}$ normal)	Complete agglutination.
4	—	—	0·75	$10^{-5.51}$ normal ($30 \cdot 7 \times 10^{-7}$ normal)	,, ,,
5	—	—	0·85	$10^{-4.54}$ normal (287×10^{-7} normal)	Dispersed.
6	—	—	1·0	$10^{-4.09}$ normal (803×10^{-7} normal)	,,
7	Na_2SO_4	·005	0·5	$10^{-6.56}$ normal ($2 \cdot 77 \times 10^{-7}$ normal)	,,
8	,,	,,	1·0	$10^{-5.20}$ normal ($63 \cdot 5 \times 10^{-7}$ normal)	Complete agglutination.
9	,,	·05	0	$10^{-7.00}$ normal ($1 \cdot 00 \times 10^{-7}$ normal)	Dispersed.
10	,,	,,	0·5	$10^{-6.42}$ normal ($3 \cdot 81 \times 10^{-7}$ normal)	,,
11	,,	,,	1·0	$10^{-5.51}$ normal ($30 \cdot 6 \times 10^{-7}$ normal)	,,

equivalent amount of chlorine remained behind in the solution as free acid so that the reaction of the solution was materially altered. Lewis (28) obtained evidence indicating that, in the case of an emulsion of petroleum oil precipitated with barium chloride, barium was adsorbed on the particles of oil in excess of chlorine. In all these cases agglutination is associated with discharge of the particles.

If the adsorption of ions were to continue in excess of the amount required to discharge the particles, they would take on the opposite charge with the corresponding lowering of surface action. The particles would then again repel one another and dispersion would ensue. This has actually been shown to occur by Burton (29), who found that colloidal copper particles, which carry a positive charge, will migrate to the anode after addition of excess of potassium phosphate to the solution. Reasoning from this analogy, the explanation, for example, of the behaviour of egg-white proteins towards sodium citrate in acid solution, would be that the negatively charged anions when present in minimal concentration are able to discharge the positive charge carried by the protein particles; if, however, the concentration of the citrate is increased, the transaction is overdone owing to the powerful charge carried by the trivalent anion, and the protein, first becoming discharged, is afterwards re-charged with a sign opposite to that originally held[1]. The difference between denaturated serum- and egg-proteins then becomes one of degree and not of kind. Dispersed particles of the latter are readily discharged but with difficulty overcharged; in the case of the former the tendency to become overcharged is so great that electrically neutral particles are obtained with difficulty if electrolytes are present in more than minimal concentration, especially when these are composed of multivalent ions.

The colloidal particles of proteins would seem to be extraordinarily sensitive to this action of electrolytes and to display a quite peculiar tendency to adsorb ions. To test this hypothesis it was necessary to compare the sign of the electric charge on protein particles before and after dispersion by salts. The direction of migration in an electric field was observed under a microscope with a dark ground illumination. For this purpose the degree of dispersion must not be too great or the particles become invisible with the ultra-microscope. The liquid was placed in a shallow glass cell built upon a microscope slide

[1] The observations of Mines (30) are also of the same order; he has shown that the red blood-cells of *Scyllium canicula* are clumped by low concentrations of cerium chloride and again dispersed when the concentration of the salt is raised, the cells acquiring an electric charge of reverse sign to that originally carried.

(Fig. 4). The sides of the cell were formed by two slips of cover-glass ·3 mm. in thickness cemented upon the slide with balsam. The ends of the cells were composed of two pieces of platinum, of the same thickness, similarly cemented on to the slide. The roof of the cell was formed by a thick cover-glass. One of the pieces of platinum was a little shorter than the other so as to permit of a small exit channel for the excess of liquid when the cover-glass was placed in position. To each of the pieces of platinum foil a spiral of platinum wire was welded which served to convey the current from the accumulators. The electrodes were 2 cm. apart and the difference of potential employed was 8 volts. In using the method, in order to avoid the disturbing effects of electrolysis it is essential that the circuit be only momentarily closed whilst making the observations. The current is then reversed and the observation repeated. If it is desired to measure the velocity of movement this can be done by the aid of an ocular micrometer previously standardised for the particular optical combination used. The dispersion must be a very dilute one or too many particles will be present.

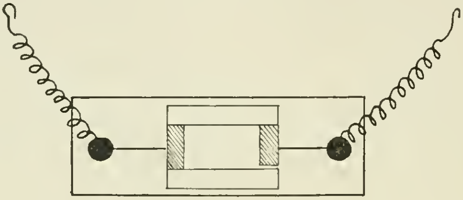

Fig. 4.

In using this method the particles observed must be situated some distance from the surface of the glass cover or slide, as in the neighbourhood of the glass the liquid moves under the influence of electric endosmose and of course carries the particles with it. With the electric field we employed, 4 volts per cm., this effect was confined to a depth of ·05 mm. from either surface. Another limitation of the method is that if the concentration of electrolyte is above a certain limit, gas bubbles arise at the electrodes and cause movements of the particles. The range of concentration of electrolyte with which it is possible to work without formation of gas is considerably extended by covering the electrode with platinum black.

In the case of serum, it is seen from Table XIV that dispersion caused by sodium citrate and sodium sulphate in acid solution was accompanied by a change of sign in the charge carried by the protein particles from positive to negative. That this effect is not dependent upon reduction in hydrogen-ion concentration is seen in Table XVIII, where particles of denaturated serum-protein dispersed by Na_2SO_4 carry a negative charge in a solution which is acid of the iso-electric point.

In alkaline solution the dispersed particles with their – charge were agglutinated and afterwards dispersed by $CaCl_2$ and $La(NO_3)_3$ but the possession of a + charge was only demonstrated in the case of the latter salt. The colloidal solution with $CaCl_2$ is difficult to explain as we were not able to demonstrate the existence of any charge on the dispersed particles (see Table XIV). The solution of denaturated egg-white by $CaCl_2$ solution is also anomalous, seeing that no dispersion was obtained with $La(NO_3)_3$ which from all analogy should have had a more powerful effect. In the case of the dispersion by $CaCl_2$ the concentration of electrolyte required was too great to make any satisfactory observations upon the charge carried. A change of sign in the latter was however demonstrated in the case of egg-white and sodium citrate. The particles were previously dispersed in acid solution with a positive charge, and on addition of sodium citrate were at first precipitated. With higher concentration of the salt the particles were again dispersed, this time bearing a negative charge (see Table XIII).

TABLE XVIII. *Influence of Na_2SO_4 upon the charge carried by and agglutination of particles of denaturated serum-protein and upon the hydrogen-ion concentration of the solution.*

Exp.	Salt added	Concentration of salt added, in terms of normality	C.c. N/100 HCl (or equivalent) added in total vol. of 10 c.c.	Concentration of H+ ions, in terms of normality	Sign of electric charge on particles	Degree of agglutination
1	—	—	·1	$10^{-6\cdot42}$ normal ($3\cdot77 \times 10^{-7}$ normal)	...	Partial agglutination.
2	—	—	·15	$10^{-6\cdot23}$ normal ($5\cdot86 \times 10^{-7}$ normal)	...	Almost complete agglutination.
3	—	—	·20	$10^{-4\cdot51}$ normal (310×10^{-7} normal)	...	Complete agglutination.
4	—	—	·50	...	+	Dispersed.
5	—	—	·70	$10^{-3\cdot24}$ normal (5780×10^{-7} normal)	+	,,
6	Na_2SO_4	·03	·30	$10^{-5\cdot45}$ normal (352×10^{-7} normal)	...	Almost complete agglutination.
6	,,	·07	·70	$10^{-4\cdot01}$ normal (970×10^{-7} normal)	–	Dispersed.

We have not been able to determine wherein lies the cause of the extra tendency to overcharge in the case of serum-proteins. It is conceivable that the charge carried by the particles may be small in amount and easily overbalanced or it need only be very small in order to keep the particles dispersed from one another. On the other hand, serum-proteins may be able to adsorb ions more readily than egg-proteins.

The sensitiveness of agglutination to minute variations in reaction and salt-content explains why it is so difficult to entirely separate serum-protein by heating the solution. It is practically impossible to perfectly adjust the reaction so that the whole of the protein is precipitated. The finding of more or less non-coagulated protein serum by Chabrié (31), Howell (32), and others is no doubt to be explained in this way.

(3) *Influence of temperature.*

(*a*) *Horse-serum.* The same solution of denaturated serum was used as in the experiments made to investigate the effect of salts given in Tables IX and X.

Convenient material for study, *i.e.* one which failed to agglutinate at laboratory temperatures and did so at a suitable rate for study at higher temperatures, was the following: a 3·3 % suspension of denaturated horse-serum, dialysed, containing in 100 c.c. 3·7 c.c. N/10 acetic acid and 1 gram NaCl in Exp. 1, and 4·4 c.c. N/10 acetic acid and ·5 grm. Am_2SO_4 in Exp. 2. Tubes containing these mixtures were placed in thermostats at different constant temperatures and agglutination-rate was measured by observing the time which elapsed before visible particles appeared.

The results of Exps. 1 and 2, set forth in Tables XIX and XX, show that there is a critical temperature below which agglutination either does not take place at all or so slowly as to be negligible for our purpose. Immediately above this point, temperature exerts a very great effect on agglutination-rate; on raising the temperature still further the influence of a definite rise in temperature becomes gradually less and less until, at temperatures far removed (30°–40° C.) from the critical temperature, the effect becomes consistent, and the temperature coefficient of agglutination settles down to a value of about 2·5 per 10° C. rise of temperature. This is shown graphically in Fig. 5, where logarithms[1] of agglutination-rate (as measured by the reciprocals of the time, in minutes, before visible particles could be detected) are plotted against temperature. In the neighbourhood of the critical temperature the curves (*a*) Exp. 1

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[1] Logarithms are plotted, as the effect in the region of the critical point is so enormous that the numbers themselves could only have been set out on a very small scale.

and (*b*) Exp. 2, rise steeply; above temperatures of about 60° C. in either case the curves tend to form straight lines, showing that the effect of temperature at this point is becoming consistent.

As there is no reason to suppose that temperature would greatly modify either the surface tension between particles and fluid or the charge on the particles, we suggest the following explanation to account for these facts. The surface energy which brings about aggregation is potential until the particles come nearly into juxtaposition. They are brought into the necessary close relationship with one another by their own movement or the movement of the fluid, the former, or Brownian movement, being the more important in the case of very fine particles. If the particles carry an electric charge, their velocity of movement will be diminished as soon as they are brought within the range of the electrical repulsion, and unless the momentum is adequate to counteract this repulsion, the particles will never arrive sufficiently close to one another for the effect of surface energy to be manifested. On this view

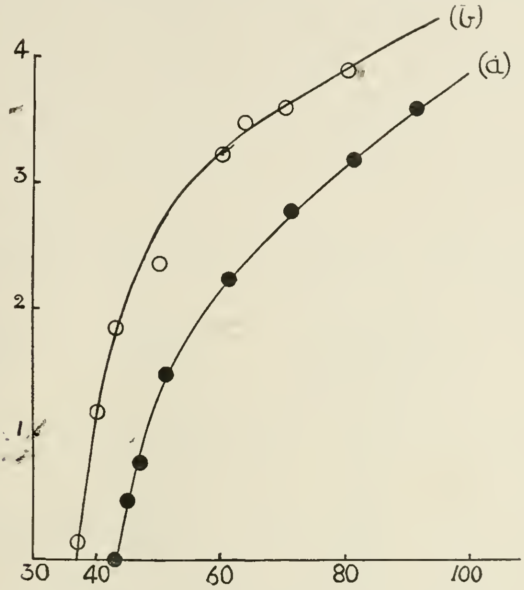

Fig. 5. Effect of temperature upon agglutination of "denaturated" diluted serum (3·3 %) containing:

(*a*) 1·0 grm. NaCl and 3·7 c.c. N/10 acetic acid in 100 c.c.
(*b*) 0·5 grm. Am_2SO_4 and 4·4 c.c. N/10 acetic acid in 100 c.c.
Ordinates = $\log_{10}$ (agglutination-rate × 10^3). See Tables XIX and XX.
Abscissæ = temperature, degrees Centigrade.

TABLE XIX. *Exp.* 1. *A suspension of denaturated diluted serum,* 3·3 $^0/_0$, *dialysed, containing in addition* 3·7 *c.c.* 1/10 *N acetic acid in* 100 *c.c. and NaCl to a concentration of* 1 $^0/_0$.

Temperature, °C.	Time elapsing after mixing before particles were visible, mins. = *t*	Agglutination-rate, as measured by *I/t*	Increase in agglutination-rate for a rise in temperature of 10° C.
43	780	0·001	...
45	390	0·003	
47	180	0·006	...
51	36	0·03	
61	6	0·17	... ... 3·7
71	1·62	0·61	... ... 2·5
81	0·65	1·54	... ... 2·6
91	0·25	4·0	

TABLE XX. *Exp.* 2. *A suspension of denaturated diluted serum,* 3·3 $^0/_0$, *dialysed, containing in addition* 4·4 *c.c.* 1/10 *N acetic acid in* 100 *c.c. and* Am_2SO_4 *to a concentration of* ·5 $^0/_0$.

Temperature, °C.	Time elapsing, after mixing before particles were visible, mins. = *t*	Agglutination-rate, as measured by *I/t*	Increase in agglutination-rate for a rise of 10° C.
37	> 360 and < 1020	...	...
40	67	0·015	
43	14	0·071	...
50	4·2	0·23	
60	0·58	1·72	... ... 2·3
63·5	0·33	3·00	
70	0·25	4·00	... ... 2·0
80	0·125	8·00	

there exists a "critical velocity" necessary for agglutination and, as the velocity of the particles is a function of temperature, an explanation is afforded of the observed critical temperature below which agglutination does not occur.

We interpret the high temperature coefficient just above the critical temperature and the subsequent falling off of the temperature effect as follows: At any given temperature we assume the degree of intrinsic energy possessed by the protein particles to be distributed among them in the manner illustrated in Fig. 6, which represents the normal frequency curve. Suppose the curve *I* to express the distribution of momentum among the particles at temperature t_1; if *A* and *B* indicate any two values of this intrinsic energy, the volume of the rectangle *Aa bB*, compared with the volume included between the curve and the base line, represents the proportion of particles possessing a kinetic energy which is greater than *A* and less than *B*. If now *C* represents the critical momentum which gives rise to a velocity sufficient to overcome the existing forces of repulsion between particles, it is seen that at temperature t_1 the material in question will contain practically no particles possessing the required critical velocity. Agglutination, therefore, will only occur with extreme slowness.

As the temperature is raised and the mean value of the particular energy is consistently increased, the distribution curve, while maintaining the same form, will be moved as a whole towards the right. Suppose curve II is the frequency curve at a higher temperature t_2. At this temperature the number of particles, *N*, which possess more than the critical velocity, represented by the volume *CcD*, will be relatively important and agglutination will now begin to be apparent.

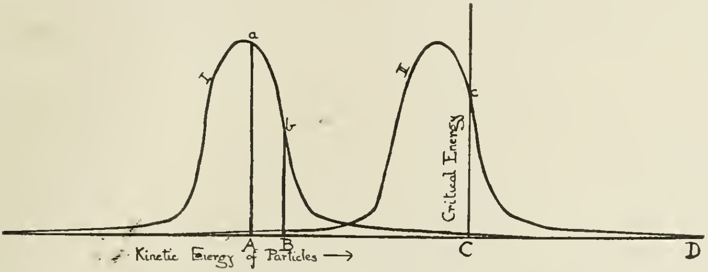

Fig. 6. Illustrating the distribution of kinetic energy among protein particles in colloidal solution at different temperatures.
Ordinates = distribution of particles possessing kinetic energy corresponding to abscissæ.

A further rise in temperature from this point onwards will cause a disproportionate increase in the value of *N*, for while with small difference in temperature, the curve as a whole may be displaced only a short distance to the right, the area *CcD*, which represents the value of *N*, will be relatively much increased. In the range of temperature immediately above the critical point the effect of temperature will therefore appear to be greatly enhanced.

At higher temperatures still, where the crest of the frequency curve has reached a point to the right of *C*, an increasing majority of the particles will possess more than the critical momentum, until, at temperatures at which all the particles possess a velocity greater than the "critical velocity," the effect of temperature will merely increase the mean value of the momentum of the particles, and will settle down to the conventional value.

THE SUPPOSED CONVERSION OF ALBUMEN INTO GLOBULIN BY HEATING IN SLIGHTLY ALKALINE SOLUTION.

We have already mentioned (p. 262) the conclusion of Starke (7), viz. that on heating a solution of egg-albumen in its natural slightly alkaline reaction, the whole of the albumen was converted into globulin. Moll (8) arrived at a similar conclusion from his experiments with serum-albumen and showed that the precipitability of serum by means of Am_2SO_4 became altered, inasmuch as the englobulin and pseudoglobulin fractions (Pick) were increased at the expense of the albumen. Starke based his interpretation upon the facts that egg-albumen so treated was precipitated by dialysis, by dilution and by acidification, and could be dissolved readily by slight excess of acid or alkali. It could be salted out by saturation with sodium chloride, potassium chloride or magnesium sulphate and, like globulin, contained less sulphur than the original albumen. Starke regarded the material as distinct from "denaturated" albumen because on heating a suspension of his supposed globulin in the presence of salts (NaCl, $CaCl_2$ or $MgSO_4$) it underwent "heat coagulation" and became converted into a firm and coherent clot which, in contradistinction to his manufactured globulin, he found to be insoluble in ·05 % sodium carbonate or in weak acid.

The observations of Starke and Moll amount to this. If a solution of albumen be heated to 60°–85° C. in presence of a small concentration of alkali, the albumen is changed into a body possessing the "solubilities" and some other properties of globulin. This body is precipitated from the solution by adjusting the reaction so as to be slightly acid (iso-electric point). On heating a similar solution of albumen rendered just acid a coherent precipitate is obtained which appears to be insoluble in water and dilute acids or alkali. The difference depends upon whether the reaction be made acid before or after heating. There is no doubt about the accuracy of the observations, but we do not believe the facts warrant the interpretation put upon them by these authors. The difference in the ease with which the two precipitates can be dissolved (or, more correctly, dispersed) by acids and alkali is, we believe, bound up with the different degree of aggregation of the particles in the two precipitates, and we see no reason to suppose that Starke's "globulin" is other than "heat-denaturated" albumen. We find that the firm coherent precipitate of egg-albumen or serum-albumen formed by boiling in acid solution can be dispersed completely although slowly by alkali or acid, the amount required depending on the concentration of the protein (1/100th normal alkali for a concentration of ·5 $^0/_0$ protein), if the particles are first mechanically separated, and a few days allowed for the process to take place.

It seems to be merely the differing degree of cohesion of the particles which determines the different solubility of the material. From what has gone before it is clear that the dispersion of denaturated albumen depends upon the possession of an electric charge, whence it follows that the difficulty in dispersing the particles of a curdy, coherent precipitate is due to the difficulty of charging them.

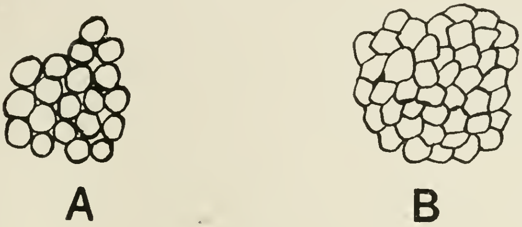

Fig. 7.

A and *B*, Fig. 7, represent the particles in a fragment of loosely agglutinated and coherent cheesy precipitate of denaturated protein respectively, as seen under the ultra-microscope. We suggest that the reason *A* disperses readily and *B* only slowly on addition of weak acid or alkali is that, in the former case, the charge is situated on the surface of the nearly discrete particles, whereas in the latter case the coherent aggregate is charged as a whole. In *A* a small charge is sufficient to tear the particles asunder, whereas in *B* any disruptive action or effect on surface tension is limited to the superficial particles, and even here operates at great mechanical disadvantage.

SUMMARY.

1. As shown by Hardy, Michaelis and others, dispersion of denaturated protein by small amounts of acid or alkali is due to the electric charge given to the particles. If this electric charge is neutralised, and the proteins become iso-electric with the solution, agglutination occurs.

2. In common with other observers, we find that reaction of the solution is the principal factor in determining the degree of agglutination of denaturated proteins.

3. The optimum acidity for precipitation in absence of electrolytes is found to be at a concentration of hydrogen-ions equal to about 3×10^{-6} normal in case of the denaturated protein of both serum and egg-white, thus confirming the figure obtained by Michaelis and Rona for the iso-electric point of serum-albumen.

4. Agglutination is greatly influenced by the presence of neutral salts. Their action is twofold:

(*a*) Alteration of the reaction of protein-containing solutions. The concentration of hydrogen-ions is lowered in acid solution and that of hydroxyl-ions in alkaline solution.

(*b*) Neutralisation or increase of the electric charge carried by the protein particles, according as the charge on the protein is of opposite or similar sign to that carried by the more potent ion of the salt.

5. In case of egg-white, agglutination of the denaturated protein is assisted by the addition of electrolytes and the range of hydrogen-ion concentration over which agglutination occurs is extended.

6. In the case of serum-proteins a similar result is obtained only when salts are present in very low concentration; in presence of more concentrated electrolytes agglutination is hindered if not altogether prevented.

7. The cause of dispersion by salts appears to be the adsorption of ions by the denaturated particles of protein. If the charge of the more potent ion is of opposite sign to their own charge they will after being first discharged, become re-charged with a sign opposite to that they originally carried. The effect is increased with higher valency.

Denaturated serum-protein is easily dispersed by electrolytes but with egg-albumen dispersion accompanied by reversal of charge was detected only after the addition of sodium citrate.

In the case of dispersion by divalent kations such as Ca^{++} we were not able to demonstrate the existence of any charge on the dispersed particles.

8. For every solution containing denaturated protein there is a critical temperature depending on the reaction and on the concentration of protein and electrolytes, below which agglutination does not take place.

9. Immediately above this critical point, rise of temperature has at first a very marked effect in enhancing the rate of agglutination; this influence, however, becomes less and less, until at temperatures far removed from the critical temperature, the effect is consistent, agglutination-rate being increased about 2–5-fold per rise of 10° C. An explanation is suggested.

10. No support is found for the conclusion both of Starke and Moll that albumen, when heated in alkaline solution, is converted into globulin; between (*a*) the dispersed protein, and (*b*) the coherent precipitate obtained on heating in alkaline and faintly acid solutions respectively, there appears to be no difference which cannot be accounted for by the different state of aggregation. Reasons are adduced for regarding the substance studied by these observers as merely heat-denaturated protein, obtained in a loose state of aggregation by the method employed.

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Die Viskosität von Kasein-Solen.

(Eingegangen am 6. August 1912)

Von Harriette Chick und Charles J. Martin (London)[1].

Das zu den vorliegenden Untersuchungen verwendete Material wurde aus einem Muster von käuflichem Kasein hergestellt, welches alkalisch reagierte und aus Natriumkaseinogenat bestand. Eine verdünnte Lösung wurde durch Auslaugen mit Wasser hergestellt, und zur Entfernung von Fett durch ein Berkefeld-Filter filtriert; das Kasein wurde weiter gereinigt durch Ausfällung mit Säure, Abfiltrieren, Waschen und Pressen des Niederschlags, welcher dann in verdünnter Natronlauge gelöst wurde. Die so erhaltene Lösung wurde durch Erhitzen im siedenden Wasserbade sterilisiert und enthielt 9,39 Proz. Natriumkaseinogenat (Trockengewicht).

Aenderung der Viskosität mit der Konzentration. Eine Reihe von verdünnten Lösungen wurde aus der oben erwähnten konzentrierten Ausgangslösung durch Zusatz verschiedener Mengen von destilliertem Wasser hergestellt; die hierbei nötigen Messungen wurden sämtlich durch Wägung ausgeführt.

Die Viskosität der verschiedenen Verdünnungen, verglichen mit der des Wassers, wurde in üblicher Weise durch Bestimmung der Durchflußzeit für ein konstantes Volumen im Ostwald'schen Viskosimeter gemessen. Sämtliche Messungen wurden im Thermostaten bei einer Temperatur von 25 °C ausgeführt. Die Dichten der verschiedenen Lösungen wurden ebenfalls bestimmt, und die entsprechenden Korrekturen bei Berechnung der auf Wasser bezogenen Viskositäten angebracht. Die Resultate sind numerisch in Tabelle I und graphisch in Fig. 1 dargestellt: die Kurven I und II in letzterer stellen die Aenderung in der Dichte, resp. der Viskosität, bei verschiedenem Gehalte an Kasein dar. In zwei Fällen, in welchen keine der Viskositätsbestimmung direkt entsprechende Dichtemessung ausgeführt worden war, wurde die letztere aus der abgerundeten Kurve I interpoliert.

Die Kurve II zeigt den raschen Anstieg der Viskosität bei zunehmendem Gehalt an Kasein, im scharfen Gegensatz zu dem angenähert linearen Anwachsen, welches bei wahren Lösungen auftritt. Die wahrscheinlichste Erklärung dieses raschen Anstieges im vorliegenden Falle ist wohl die, daß eine Verschiebung des Phasenverhältnisses eintritt, wenn wachsende Mengen von Kasein in das Sol eingeführt

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[1] Deutsch von E. Hatschek, London.

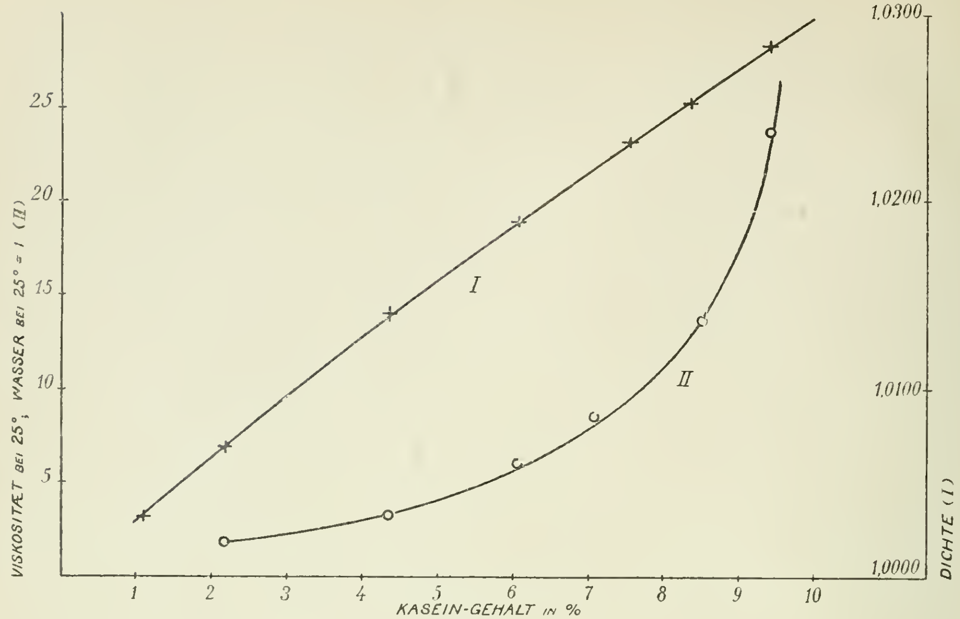

Fig. 1

Tabelle 1
Aenderung der Viskosität des Natriumkaseinogenat-Sols mit der Konzentration.
Temperatur konstant 25° C

Gehalt an Na-Kaseinogenat in Proz.	Durchflußzeit in Sekunden	Dichte des Sols[2]	Viskositäts-Koeffizient[4]
9,39	625,2	1,0283	23,72
8,49	360,6	1,0257[3]	13,66
7,06	225,0	1,0218[3]	8,48
6,05	162,8	1,0190	6,12
4,35	90,0	1,0140	3,37
2,17	48,9	1,0070	1,82

werden. Wie E. Hatschek[5] gezeigt hat, ist die Viskosität einer Suspension unabhängig von der Teilchengröße und wächst einfach linear mit dem Verhältnisse disperse Phase : Gesamtvolumen. Kasein ist als ein hydrophiles Kolloid anzusehen, welches Wasser in einer, wahrscheinlich der Quellung analogen Weise, an sich bindet. Unter dieser Voraussetzung würde, bei zunehmendem Gehalte an Kasein, sowohl das relative wie das absolute Volumen von ungebundenem Wasser, daß ist von geschlossener Phase, schneller abnehmen, und die Viskosität demnach auch rascher anwachsen, als im linearen Verhältnisse zum Kaseingehalt. Ein Anstieg auf den 13, resp. 23fachen Wert schließt den Suspensoidcharakter überhaupt aus: hier müssen die — deformierbaren — Teilchen der dispersen Phase bereits in gegenseitiger Berührung sein, was den üblichen Vorstellungen über die Struktur der Emulsoide entspricht.

Die Kurve ~~II~~, Fig. 1, welche die Aenderung der Dichte mit der Konzentration darstellt, ist gegen die Abszissen- (Konzentrations-) Achse etwas konkav. Die Dichte ist demnach nicht genau proportional dem Gehalte, was darauf hindeutet, daß bei der Dispersion des Kaseins in Wasser eine geringe Kontraktion stattfindet. Dieses Phänomen ist übrigens auch bei anderen Proteinen beobachtet worden, und wir hoffen, die Resultate der bezüglichen Messungen in einer weiteren Arbeit mitzuteilen. Im vorliegenden Falle wurde die Vermutung, daß eine Kontraktion stattfindet, in folgender Weise bestätigt. Eine direkte Bestimmung der Dichte von gepulvertem, trockenen Kasein in Benzol gab als Mittelwert zweier Messungen den Wert 1,318 (Wasser bei 4° C = 1,000). Eine Lösung von 7,85 Proz. (trockenem) Kasein in verdünnter Natronlauge hatte die Dichte 1,0240, woraus sich die Dichte des Kasein-Natriumsalzes zu 1,42 berechnet. Der Aschengehalt war 4,33 Proz. des Trockengewichtes, was einem Natriumgehalt on ungefähr 2 Proz. entspricht, vorausgesetzt, aß diese Asche aus Na_2CO_3 besteht. Unter erücksichtigung dieses Natriumgehaltes finden ir, daß die durch direkte Wägung am trockenen asein gefundene Dichte 1,316 ist, während er, aus der Dichte der Lösung berechnete ert größer ist, nämlich ungefähr 1,39.

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[2] Bezogen auf die Dichte des Wassers bei derselben Temperatur, als Einheit.

[3] Aus der Kurve I, Fig. 1., interpoliert.

[4] Wasser bei 25° als Einheit; Durchflußzeit 27,1 Sekunden.

[5] E. Hatschek, Koll.-Zeitschr. 7, 301 (1910); vgl. auch M. Bancelin, Koll.-Zeitschr. 9, 154 (1911).

Aenderung der Viskosität mit der emperatur. Tabelle II gibt die Resultate iner Reihe von Messungen, welche an der onzentrierten Lösung (mit 9,39 Proz. Natrium-aseinogenat) bei Temperaturen von 0 bis 100° C usgeführt wurden. Diese Messungen wurden der bereits beschriebenen Weise ausgeführt, nd die Werte in der Tabelle geben die Viskosität bezogen auf die von Wasser bei enselben Temperaturen. Im Hinblick uf diese Darstellungsweise hielten wir eine orrektion für Dichte für unnötig, da die relative enderung in der Dichte bei Wasser und asein-Sol jedenfalls sehr gering ist. Die Werte ür die Viskosität des Wassers wurden ebenfalls nit demselben Apparate bestimmt, und die zum ergleiche mit den Kasein-Solen notwendigen ahlen einer, die ersteren darstellenden Kurve ntnommen. Die Temperatur-Viskositätskurve st in Fig. 2 ersichtlich, wobei nochmals darauf ingewiesen sei, daß die Ordinaten stets das Verhältnis: Viskosität des Sols : Viskosität von Wasser bei derselben Temperatur darstellen.

Tabelle II

Aenderung der Viskosität eines 9,39 prozentigen Natriumkaseinogenat-Sols mit der Temperatur.

Temperatur °C	Durchflußzeit in Sekunden a) Kasein-Sol t	 b) destilliertes Wasser t_w	Viskositätskoeffizient, bezogen auf Wasser bei derselben Temperatur, d. i. $\frac{t}{t_w}$
0.00	3830,0	59,0	64,9
9,75	1916,0	39,4	48,6
23,0	702,6	28,2	24,9
30,6	463,6	24,5	18,9
40,0	299,6	21,0	14,3
51,7	193,0	17,9	10,8
60,2	147,0	16,2	9,08
72,7	103,0	14,2	7,26
81,25	82,4	13,2	6,24
99,6	56,1	12,1	4,64

Die Betrachtung dieser Resultate legt den Gedanken nahe, daß die Aggregate des Kaseinsalzes bei steigender Temperatur das gebundene Wasser freigeben, so daß die Viskosität, dem sehr rasch anwachsenden Volumen an geschlossener Phase entsprechend, schnell abfällt.

Wie zu erwarten steht, wird diese Erscheinung bei abnehmendem Kaseingehalt nach und nach weniger ausgesprochen. So zeigte z. B. ein Sol mit einem Gehalte von 1,6 Proz. Natriumkaseinogenat bei steigender Temperatur (von 17 bis 50°) einen Viskositätsabfall, der nur wenig — 1,18 mal — größer war, als die entsprechende Abnahme bei Wasser.

Saure und alkalische Sole. Wir unternahmen auch noch einige Versuche, bei welchen die Kasein-Sole einen zunehmenden Zusatz an Säure und Alkali erhielten, und fanden einen entsprechenden Anstieg der Viskosität in beiden Fällen. Dies bestätigt die Befunde von E. Laqueur u. O. Sackur[6]). Die genannten Forscher schließen aus dieser Tat-

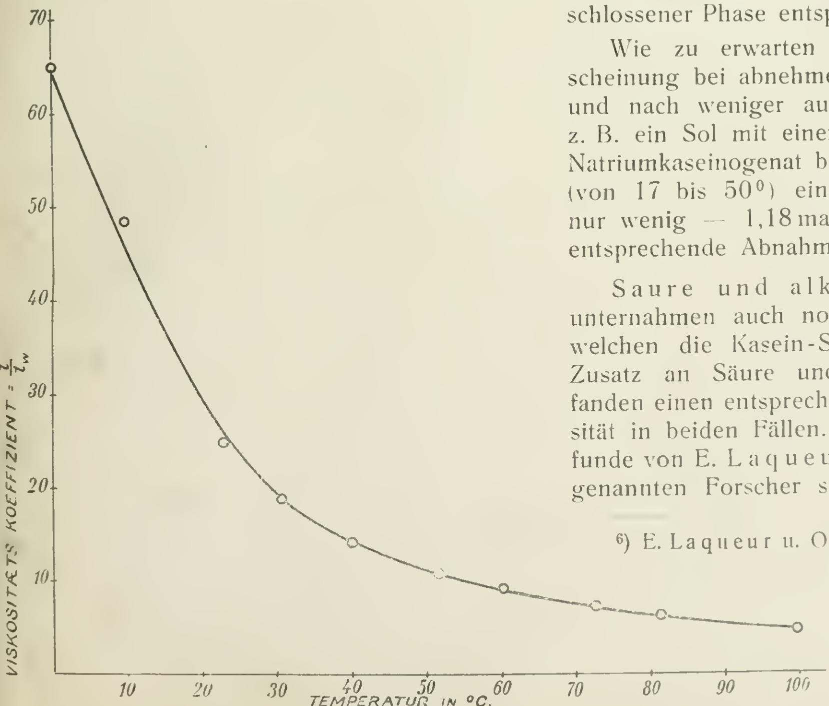

Fig. 2

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[6]) E. Laqueur u. O. Sackur, Beitr. zur chem. Phys. u. Path. **3**, 193 (1903); ein ähnliches Phänomen bei Serum beobachteten Wo. Pauli u. H. Handovsky, Biochem. Zeitschr. **18**, 340 (1909); vgl. auch H. Chick u. Ch. J. Martin, Jnl. of Physiology **45** (1912).

sache, daß die innere Reibung des Kasein-Sols — entsprechend seiner Natur als amphoteres Protein — durch Ionisierung anwächst, d. i. als Folge der elektrolytischen Dissoziation des durch Alkali- resp. Säurebindung gebildeten Salzes. Wir möchten dagegen die Erscheinung derart erklären, daß die Aggregate von Kaseinsalzen ein höheres Wasserbindungsvermögen besitzen, und daß demnach eine Verschiebung des Phasenverhältnisses stattfindet, wobei das Volumen an disperser Phase auf Kosten der geschlossenen Phase anwächst, mit einem entsprechenden Anstieg der Viskosität.

Zum Schlusse sprechen wir unseren besten Dank Herrn E. Hatschek aus, auf dessen Anregung diese Arbeit unternommen wurde und dessen Rat und Unterstützung uns stets zur Verfügung standen.

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VIII. THE DENSITY AND SOLUTION VOLUME OF SOME PROTEINS.

By HARRIETTE CHICK and CHARLES JAMES MARTIN.

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(*Received November 25th, 1912.*)

In a previous communication dealing with the viscosity and other properties of caseinogen solutions [1912], we pointed out that there was a shrinkage in volume and corresponding increase in density when that protein formed colloidal solution. In order to investigate the point the method employed was to determine the density of the dry, powdered protein by weighing in benzene or some other indifferent fluid and to compare the value with that obtained by calculation from the observed density of a fairly concentrated solution. The latter value was found to be much greater than the former.

As far as we have been able to ascertain, this is the first attempt to determine the density of proteins with the exception of gelatin. Quincke [1903] found the specific gravity of a specimen of the latter to be 1·368, while Lüdekings [1888] had previously given the value of 1·412, and had shown that the density calculated from that of jellies (14 to 35 % gelatin) was as high as 1·9.

The increase in density when proteins are dissolved in water is an instance of the phenomenon constantly associated with colloidal solution of the lyophile or emulsoid character. The intimate association of the protein particles with water leads to a shrinkage in total volume, so that the increase in density is more properly regarded as an attribute of the whole system than of the protein alone. Analogous cases are those of gum-tragacanth and starch. In the case of the latter Rödewald [1897] found the density in the dry state and that calculated from that of a solution to be 1·38 and 1·49 respectively.

In our experiment with caseinogen, a sample of the pure, dry, powdered protein was prepared by acidifying a solution in dilute sodium hydroxide, washing and drying the precipitate, and grinding to a fine powder. A known weight was inserted in a pyknometer bottle, covered with benzene of known density, and all the air expelled by careful heating *in vacuo*. The bottle was then filled up with benzene in a thermostat at 25° C. and weighed. The weight obtained was compared with that of the bottle filled with benzene at the same temperature, and the density of the powder calculated. The value obtained was 1·318 as the mean of two determinations (see Table I). A solution of caseinogen (7·85 %) in dilute sodium hydroxide gave a density of 1·0240, from which the density of sodium caseinogenate was calculated to be 1·42.

In order to compare this value with that obtained by direct determination, it is necessary to apply a small correction, seeing that the solution consisted of sodium caseinogenate. The ash present composed 4·331 % of the dry weight and, assuming this to be sodium carbonate, 2 % of the dry weight would consist of sodium, and the density, for comparison with that of the powdered caseinogen, should be reduced by that amount. After this correction has been made the caseinogen in solution is still seen to be denser than the solid caseinogen in the proportion of about 1·39 to 1·318.

In addition to caseinogen three other pure proteins have been similarly investigated, viz.:

Crystalline serum albumin, prepared from horse-serum by the method of Hopkins and Pinkus [1898].

Serum globulin (pseudo-globulin). Three different samples were investigated, obtained from horse-serum by different methods. I and II were prepared by repeated precipitation with half-saturated ammonium sulphate, the euglobulin present in the serum being separated by the subsequent dialysis; in the case of III the latter was removed by preliminary precipitation with saturated brine, after which the pseudo-globulin was precipitated from the warm diluted filtrate by adding anhydrous sodium sulphate until the concentration equalled 20 % Na_2SO_4. The precipitate was thoroughly washed with a solution of sodium sulphate of the same strength.

Crystalline egg-albumin, prepared from egg-white, also by the method of Hopkins and Pinkus.

All the proteins were thoroughly dialysed in presence of toluene and filtered, and concentrated solutions were finally obtained. Determinations were then made of the protein-content and the density. The latter were obtained by pyknometer readings, carried out usually at 15° C. or 20° C., the density of water at the same temperature being taken as unity for the purpose of calculation. It was found that the proportionality between the density of water and that of the protein solution was, within our error of experimentation maintained between 15° C. and 25° C.

Dry specimens of the proteins were obtained by evaporating the solutions to dryness and grinding the residue to a fine powder. The evaporation and drying were carried out *in vacuo* at room-temperature, and were continued until the weight remained constant, an operation requiring 2–6 weeks. It was feared that, if the samples were dried at a high temperature, the denaturation of the proteins might introduce a source of error. This would not appear to be so, for, in the case of serum albumin, the densities of two samples dried at 100°–110° and 20° respectively, were not found to differ by any significant amount (Table I). The densities of the various powders were determined by weighing in benzene of known density (water at 4° C. = 1·00) at 25°, as described above.

TABLE I.

Density of Protein in solution compared with that in the solid state.

Protein	*In solution*			*Dry State*
	Concentration of protein % (by weight)	Density of the solution	Calculated density* of the protein	Density* of the protein
Caseinogen	7·85	1·0241	1·39†	1·318
Egg-albumin (crystalline) ...	14·6	1·0401	1·359	1·269‡
Serum-albumin (crystalline)	22·15	1·0647	1·378	1·275§ 1·281‡
Serum-globulin I	15·33	1·0428	1·365	1·279§
Serum-globulin II	16 35	1·0466	1·374	1·289‡
Serum-globulin III	11·05	1·0316	1·384	1·312‡

* Density of water at 4° C. = 1·00.
† Corrected for presence of sodium.
‡ Dried at room temperature *in vacuo*.
§ Dried at 105°—110° C.

The results are given in Table I and show that the density of albumin and globulin is also increased when in the state of colloidal solution, and to a higher degree (6·8 %) than was found for caseinogen (5 %). Egg- and serum-albumin have an almost identical density in the dry condition, viz. 1·269 and 1·281, and this is increased in the same proportion on entering solution, viz. to 1·359 and 1·378 respectively. The three samples of globulin give 1·293 as mean value when dry and 1·374 when in solution.

In Tables II and III are given the variation of the density of solutions of serum-albumin and serum-globulin with alteration in protein concentration. If the latter is plotted against the former, straight lines are obtained. The curve for caseinogen, on the other hand, shows a slight convexity which is rather more than can be explained by our experimental error, the contraction on entering solution being proportionally greater for dilute solutions. This is shown in the third column of Table II, where the value of the density of caseinogen calculated from that of its solution is seen to decrease progressively from 1·465 to 1·412 as the concentration of the protein is increased. In the case of the serum proteins, however, the value remains constant (Table III).

TABLE II.

Density of Caseinogen (Na Caseinogenate) solutions of varying concentration.

Concentration of caseinogen °/₀ (by weight)	Density of the solution*	Calculated density of sodium caseinogenate
9·39	1·0283	1·412
8·33	1·0250	1·409
7·52	1·0232	1·424
6·05	1·0190	1·437
4·35	1·0140	1·460
2·173	1·0070	1·465
1·086	1·0033	†

* Compared with water = 1·00 at the same temperature, 15° C.

† Solution too dilute for trustworthy calculation.

TABLE III.

Density of solutions of Horse-Serum Proteins of varying concentration.

Protein	Concentration °/₀ (by weight)	Density of the solution*	Calculated density of the protein
Serum-globulin	15·33	1·0428	1·365
,, ...	10·32	1·0290	1·374
,, ...	6·916	1·0190	1·365
,, ...	3·478	1·0096	1·370
Serum-albumin	22·15	1·0647	1·378
,, ...	15·16	1·0440	1·382
,, ...	7·725	1·0220	1·381

* Compared with water = 1·00 at the temperature of expt.

SUMMARY.

A comparison has been instituted in case of four proteins, viz. caseinogen, egg- and serum-albumin, and serum globulin, between the density directly determined with dry specimens and that calculated from the specific gravity of concentrated solutions. The latter is found to be 5–8 °/₀ in excess of the former, showing the extent of shrinkage in volume taking place when these proteins enter colloidal solution.

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ON THE RELATIONS OF PHENOL AND META-CRESOL TO PROTEINS; A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION

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In the case of such substances as formaldehyde, halogens, mercuric chloride, oxidising agents, acids and alkalies, it is possible to understand why they should behave as disinfectants, as they all form chemical combinations with proteins. We have, however, very little knowledge of the relations of phenol and the cresols to proteins, so that it is at present not possible to understand the nature of the germicidal action of these common disinfectants.

Reichel,[1] in 1909, showed that heat-coagulated serum and egg-white absorbed phenol from its aqueous solution in an amount directly proportional to the concentration and that the process was reversible. He also showed that *Bacillus pyocyaneus* absorbed phenol, and that the addition of sodium chloride increased the uptake by both bacilli and heat coagula and also increased the bactericidal action of phenol.

Herzog and Betzel,[2] in 1911, found that yeast reversibly absorbed phenol in an amount, which relatively decreased with rise in concentration. A rise in phenol concentration, on the other hand, increased the rate of disinfection of yeast-cells to a degree disproportionate to the increase in concentration. They conclude that the absorption of the germicide is the first phase in the process of disinfection, and that this is followed by the chemical action of the germicide upon the cells, which is not affected by concentration in the same way as the absorption.

The object of the investigation described below was to obtain further knowledge of the relations of phenol and m-cresol to proteins with a view to understanding the nature of their germicidal action.

THE METHOD OF STUDY

(*a*) *The estimation of phenol and cresol*

The method used was that described by Lloyd[3] in 1905. It consists in the bromination of phenol in the presence of a large excess of hydrochloric acid, under which condition three bromine atoms are quantitatively introduced into the phenol molecule.

Some further experiments showed that it was also possible to employ this method for the accurate determination of meta-cresol. Accurate results, however, could not be obtained with ortho- and para-cresol. The relative positions of the hydroxyl and methyl groups in the cresol molecule evidently influence the entrance of bromine atoms into the benzene nucleus.

(*b*) *The materials used*

The proteins used were gelatin, precipitated casein, dialysed crystalline egg-albumen and heat-coagulated egg-globulin (the fraction of egg-white precipitated by 1/2 saturated ammonium sulphate).

The gelatin (best gold label, Swiss) was first washed in running water for twenty-four hours. It was then air dried for twenty-four hours, cut into slips, and dried in a muslin bag at 110° C.

Merck's casein, prepared according to Hammarsten, was used in the experiments with casein.

The egg-albumen was prepared in the crystallized state from egg-white by the method of Hopkins and Pinkus, described in 1898,[4] and was dialysed for ten days, first of all, in tap-water and finally in distilled water. The heat-coagulum was prepared by heating the dialysed solution of the protein slightly acidified by a few drops of 1 per cent. acetic acid. The coagulum was well washed and dried at 105° C.

The globulin fraction of egg-white was separated from the albumen as far as possible by several precipitations with half-saturated ammonium sulphate. The globulin was then dissolved in very dilute ammonium sulphate, coagulated by heat, washed free of ammonium sulphate and dried at 105° C.

(c) *The experimental methods*

Experiments with gelatin.—A definite amount of gelatin in the form of slips about one inch by three inches was suspended in a known volume of an aqueous solution of phenol of estimated strength. When the maximum amount of phenol had been taken up by the protein—the equilibrium-time having been determined by some preliminary experiments—the strength of the aqueous phenol solution was again estimated. By a comparison of the initial and final concentrations the distribution of phenol between water and protein was determined. New equilibria with phenol between water and gelatin were obtained by withdrawing a known volume of the water-phase and replacing this with an equal volume of water or phenol solution.

Experiments with casein, egg-albumen and egg-globulin.—The suspended casein was so fine that it could not be completely removed by filtration. Accordingly, in the experiments with it, and also with egg-albumen and globulin, the method introduced by Moore and Bigland[5] was employed. This consists in restricting the protein to one region of the water-phase by means of a piece of dialysing paper, which was crumpled into the shape of a bag and enclosed in a stoppered bottle. Phenol was found to pass the dialyser readily. When equilibrium was attained, samples were removed for analysis from the portion of the water phase outside the dialyser. In other respects the procedure was similar to that adopted in the experiments with gelatin. All experiments were carried out at 20° C. in a thermostat, except where otherwise stated.

THE EXPERIMENTAL RESULTS

I

The Influence of Concentration upon the Distribution of Phenol between Water and Proteins

(*a*) *Gelatin*

Phenol below concentrations of 2·6 per cent. caused no visible change in gelatin. When immersed in concentrations between 2·6 and 5 per cent. the gelatin became white and semi-transparent but did not lose its shape, and in concentrations above 5 per cent. it contracted, lost its form, and settled on the bottom of the vessel as a viscous mass. The precipitated protein was soluble in hot 5 per cent. phenol solutions, but on cooling the gelatin was reprecipitated, having lost the property of setting. These changes were reversed when the supernatant liquid was poured off and replaced by water. In the case of the effects of concentrations less than 5 per cent., the protein quickly assumed its original appearance, except that it had acquired the property of swelling considerably, just as it does after immersion in acid. The reversibility was less rapid when the gelatin had been immersed in phenol concentrations above 5 per cent. The recovered gelatin was still soluble in warm water, and had regained the property of setting when the solution was cooled.

The time required for equilibrium.—The maximum amount of phenol was taken up by gelatin from concentrations less than 2·6 per cent. in two minutes. The reverse process was complete in fifteen minutes.

From concentrations of 2·6 per cent. to 5 per cent., namely, those which visibly changed the gelatin but did not prevent its suspension in water, the uptake of phenol was complete in twenty-four hours, and the reverse process was finished in the same time.

From concentrations of 5 to 7 per cent., causing the precipitation of the protein on the bottom of the vessel, the maximum amount of phenol was absorbed in forty-eight hours. The reverse process, however, required five weeks for its completion.

In concentrations from 0 to 2·6 per cent., at which point the gelatin was precipitated, the phenol was distributed between water and protein according to the partition-law. It was about three times as soluble in gelatin as in water.

At concentrations of about 2·6 per cent. the gelatin became white and opaque, and a greatly increased proportional uptake of phenol occurred.

TABLE I

EQUILIBRIA

10 grams gelatin ; 250 c.c. phenol solution

	Water-phase					
	Initial phenol concentration (grams per 100 c.c.)	Final phenol concentration (grams per 100 c.c.)	Amount of phenol in grams absorbed by 1 gram gelatin (by difference)	Condition of protein	Distribution-ratio $\frac{\text{Amount of phenol held by 1 gram gelatin}}{\text{Amount of phenol held by 1 gram water}}$	
Experiment I—						
(B)	0·990	1·039	0·026	Unprecipitated	2·5	Mean = 2·9
(B)	1·162	1·237	0·039	,,	3·2	
(A)	1·813	1·729	0·047	,,	2·7	
	2·167	1·936	0·058	,,	3·0	
	2·365	2·277	0·069	,,	3·0	
	2·801	2·609	0·117	Precipitated	4·5	
	3·077	2·847	0·175	,,	6·2	
	3·255	3·027	0·232	,,	7·7	
	3·399	3·188	0·285	,,	9·0	
	3·816	3·488	0·383	,,	11·0	
	4·272	3·938	0·484	,,	12·3	
	4·535	4·260	0·566	,,	13·3	
	5·625	4·962	0·765	,,	15·4	
	5·976	5·524	0·901	,,	16·3	
	6·283	5·910	1·012	,,	17·1	
	6·489	6·154	1·113	,,	18·1	

Experiment II (Diagram I).—1·02 grams gelatin ; 60 c.c. phenol solution

	Water-phase					
	Initial phenol concentration (grams per 100 c.c.)	Final phenol concentration (grams per 100 c.c.)	Amount of phenol in grams absorbed by 1 gram gelatin	Condition of protein	Distribution-ratio	
(A)	0·410	0·390	0·0123	Unprecipitated	3·1	Mean = 2·9
	0·575	0·551	0·014	,,	2·5	
	0·739	0·706	0·019	,,	2·7	
	1·889	1·791	0·057	,,	3·2	
	2·518	2·402	0·068	,,	2·8	
	2·665	2·566	0·075	,,	2·9	
	3·778	3·246	0·313	Precipitated	9·6	
	4·080	3·609	0·352	,,	9·7	
	5·037	4·008	0·605	,,	15·1	
	6·296	4·838	0·858	,,	17·7	
	6·561	5·510	0·970	,,	17·6	
	7·556	5·606	1·150	,,	20·5	
(B)	2·419	3·244	0·373	,,	11·5	
	0·934	2·707	0·104	Unprecipitated	3·8	Mean = 3·6
	0·540	1·122	0·031	,,	2·8	
	0·561	0·579	0·020	,,	3·4	
	0·386	0·392	0·017	,,	4·3	

N.B.—(A) Equilibria obtained whilst phenol concentration was increased

(B) Equilibria obtained by reverse process.

With further increase in concentration the distribution-ratio continued to rise. No evidence of saturation of the gelatin with phenol was obtained, although the saturation point in water was closely approached.

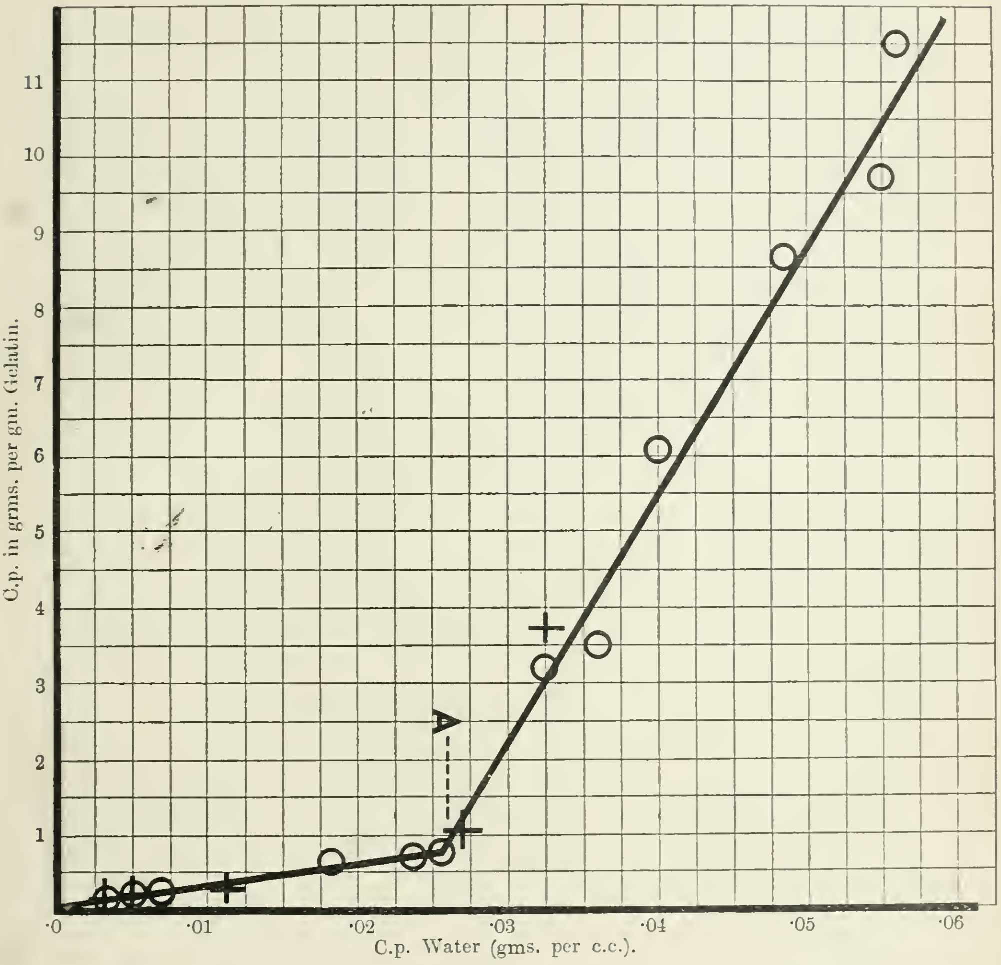

Diagram I.—Equilibria with phenol between water and gelatin showing influence of phenol-coagulation upon the distribution of phenol between water and protein. (*Experiment II*, Table I).

O = Equilibria obtained during rising concentration of phenol.
+ = Equilibria obtained by reverse process.
A = Commencement of precipitation of protein.

The equilibria (A) in Experiments I and II were obtained whilst the concentration of phenol was successively raised, and equilibria (B) by the reverse process. The approximate coincidence of the two series of equilibria, indicated by Diagram I, shows that the absorption of phenol by gelatin is a reversible process throughout the whole range of concentration investigated. The precipitation at 2·6 per cent. is also reversible.

It appears that, although unprecipitated gelatin below concentrations of 2·6 per cent. absorbs phenol according to the partition-law, when the concentration of phenol in the water reaches about 2·6 per cent. the phenol enters into a new relationship with the gelatin with the result that the protein undergoes precipitation and its absorptive capacity for phenol is enormously increased. The experiments on the distribution of phenol between water and gelatin did not afford good results when higher concentrations of phenol were employed, as equilibria were difficult to obtain. The precipitation of the gelatin in concentrations above 2·6 per cent. seems to be responsible for the somewhat erratic results. Nevertheless, the observational points plotted in diagram 1 evidently indicate again a linear relation to concentration, but in this case the uptake of phenol by the gelatin is proportional, not to the total concentration of phenol in the water-phase, but to this less 2·6 per cent. What the significance of this may be I am unable to suggest, but it is evidently associated with the sudden change in condition of the gelatin on precipitation.

(*b*) *Egg-albumen in solution*

Egg-albumen was more sensitive than gelatin to the precipitating action of phenol. A 10 per cent. suspension of gelatin in water was not visibly affected until the phenol concentration reached about 2·6 per cent., whereas a 10 per cent. solution of egg-albumen was rendered turbid by 1 per cent. phenol, slightly precipitated by $1\frac{1}{2}$ per cent., and completely precipitated by 2 per cent. phenol. Smaller concentrations of egg-albumen were affected by still lower phenol concentrations. The precipitates of egg-albumen were stable on the addition of water.

The absorption of phenol by unprecipitated egg-albumen and the reverse process were both complete within twenty-four hours. In the case of the protein precipitated by phenol the equilibrium approached from either side was attained within ninety-six hours.

TABLE II

EQUILIBRIA

	Water-phase		Amount of phenol held by 1 gram of egg-albumen in grams (by difference)	State of protein	Distribution-ratio $\frac{\text{Amount of phenol held by 1 gram protein}}{\text{Amount of phenol held by 1 gram water}}$	
	Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)				
Experiment I (Diagram II).—10 c.c. 16·47 per cent. egg-albumen solution ; 50 c.c. phenol solution						
(B)	0·251	0·237	0·005	Unprecipitated	2·1	Mean =2·2
(B)	0·332	0·312	0·007	,,	2·3	
(B)	0·805	0·757	0·025	Turbid	3·3	
(B)	1·259	1·055	0·074	Slightly precipitated	7·1	
(B)	1·666	1·332	0·122	Precipitated	9·1	
(B)	4·233	3·229	0·365	,,	11·3	
(A)	0·777	0·874	0·086	,,	9·8	
(A)	0·510	0·579	0·0614	,,	10·5	
Experiment II.—10 c.c. 9·62 per cent. albumen solution ; 50 c.c. phenol solution						
(B)	0·138	0·132	0·004	Unprecipitated	3·0	Mean =3·1
(B)	0·214	0·203	0·007	,,	3·5	
(B)	0·712	0·695	0·015	,,	2·1	
(B)	0·971	0·938	0·035	Turbid	3·7	
(B)	1·666	1·440	0·140	Precipitated	9·7	
(A)	0·600	0·701	0·077	,,	11·0	
(A)	0·292	0·344	0·045	,,	13·1	
(A)	0·143	0·174	0·025	,,	14·3	
(A)	0·102	0·114	0·018	,,	15·8	

N.B.—(B) Equilibra obtained by uptake of phenol.

(A) Equilibria obtained by reverse process.

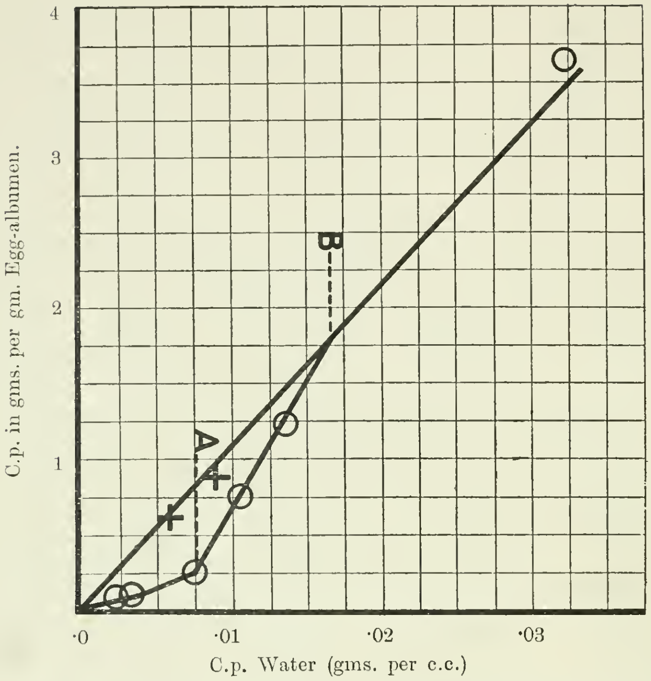

Diagram II.—Equilibria with phenol between water and egg-albumen showing the influence of phenol-coagulation on the distribution of phenol between water and protein. (*Experiment I*, Table *II*).

O = Equilibria obtained during rising concentration of phenol.

+ = Equilibria obtained by reverse process.

A = Commencement of precipitation. B = Completion of precipitation.

Experiment III.—10 c.c. 10 2 per cent. egg-albumen solution ; 50 c.c. phenol solution

	Water-phase		Amount of phenol in grams taken up by 1 gram protein (by difference)	State of protein	Distribution-ratio	
	Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)				
(B)	0·164	0·155	0·005	Unprecipitated	3·2	Mean =2·9
(B)	0·410	0·390	0·012	,,	3·0	
(B)	0·639	0·623	0·014	,,	2·3	
(B)	0·737	0·727	0·018	,,	2·5	
(B)	0·877	0·857	0·030	,,	3·5	
(B)	2·148	1·807	0·215	Precipitated	11·9	
(B)	4·926	3·466	0·376	,,	10·8	
(B)	4·651	4·235	0·460	,,	10·9	
(B)	5·475	5·280	0·575	,,	10·9	
(B)	6·056	5·742	0·760	,,	13·2	
(A)	3·346	3·812	0·486	,,	12·7	
(A)	1·444	1·699	0·226	,,	13·3	
(A)	0·708	0·864	0·134	,,	15·5	
(A)	0·360	0·441	0·086	,,	19·5	
(A)	0·184	0·231	0·059	,,	25·5	
(A)	0·096	0·119	0·046	,,	38·7	

Experiment IV.—10·2 per cent. egg-albumen solution ; 50 c.c. phenol solution

(B)	0·400	0·383	0·011	Unprecipitated	2·9	Mean =3·0
(B)	0·400	0·387	0·010	,,	2·6	
(A)	0·258	0·262	0·009	,,	3·4	
(A)	0·258	0·262	0·008	,,	3·1	

N.B.—(B) Equilibria obtained whilst phenol concentration was rising.

(A) Equilibria obtained by reverse process.

The results obtained show that the distribution of phenol between water and unprecipitated egg-albumen followed the partition-law, and that phenol was on the average 2·8 times as soluble in the protein as in water. The absorption of phenol by the egg-albumen was reversible.

A comparison of the above results with those of the experiments with gelatin indicates that the solubilities of phenol in egg-albumen and gelatin were approximately equal. Since egg-albumen was precipitated by much lower phenol concentrations than gelatin, there was therefore no relationship between their susceptibilities to precipitation and their absorptive capacities for phenol.

In the neighbourhood of 1·0 per cent. final concentration of phenol, where the precipitation of egg-albumen commenced, as indicated in Diagram II, there was a greatly increased proportional uptake of phenol, and with rising concentration the distribution-ratio continued to increase until precipitation was complete (at about 1½ per cent. final concentration). With further rise in concentration the uptake of phenol by the protein again became proportional to the concentration in the water, but with a higher partition-coefficient than at the commencement.

The observations on equilibria, made as the concentration of phenol in the water was being successively diminished, show that the egg-albumen once having been irreversibly precipitated by phenol maintains the high absorptive capacity for this substance until the concentration of phenol in the water is reduced to zero. Although with diminishing concentration the coagulated albumen gave up some of its absorbed phenol, so that in low concentrations it only retained a very small amount, the protein remained permanently denatured. It would appear that the denatured albumen is not an intimate phenol-protein complex, but is some modification of the original protein, which possesses a high solvent power for phenol.

(c) *Egg-albumen coagulated by heat*

TABLE III

EQUILIBRIA

	Water-phase: Initial concentration (grams per 100 c.c.)	Water-phase: Final concentration (grams per 100 c.c.)	Weight of coagulum taken in grams	Amount of phenol in grams absorbed by 1 gram coagulum (by difference)	Distribution-ratio Amount of phenol held by 1 gram coagulum / Amount of phenol held by 1 gram water	
	Experiment I.—25 c.c. phenol solution					
	4·916	4·054	0·5	0·431	10·6	Mean =9·8
	1·992	1·682	0·5	0·155	9·2	
	0·987	0·822	0·5	0·082	10·0	
	0·984	0·703	1·0	0·070	10·0	
	0·584	0·515	0·35	0·049	9·5	
	Experiment II (Diagram III).—25 c.c. phenol solution					
(A)	6·512	5·190	0·5	0·661	12·7	Mean =12·9
(B)	2·076	2·706	0·5	0·346	12·8	
(A)	0·495	0·399	0·5	0·048	12·0	
(B)	0·159	0·198	0·5	0·028	14·2	

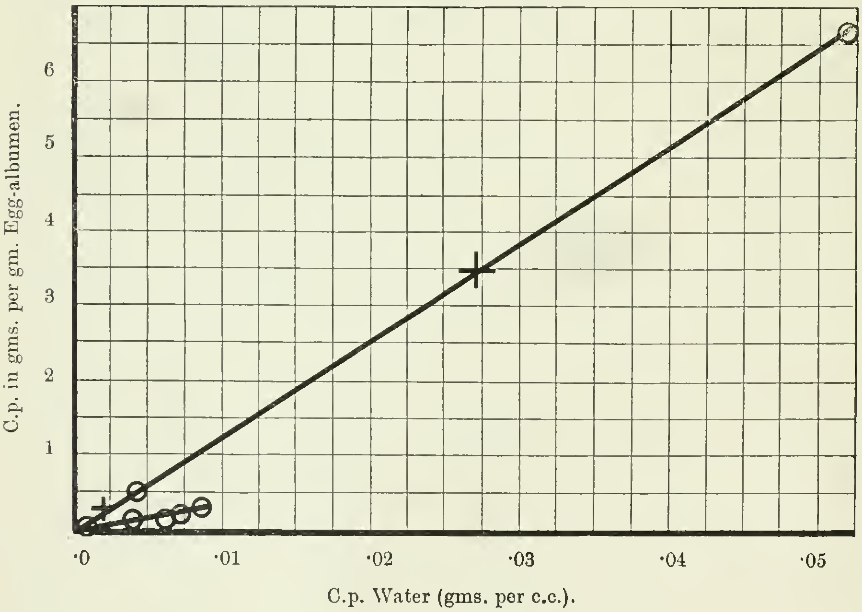

DIAGRAM III.—Equilibria with phenol between water and egg-albumen showing the influence of heat-coagulation on the distribution of phenol between water and protein.

Lower Curve.—Unprecipitated protein. (*Experiment III*, Table II).

Upper Curve.—Heat-coagulated protein. (*Experiment II*, Table III).

O = Equilibria obtained during rising concentration of phenol.

+ = Equilibria obtained by reverse process.

The distribution of phenol between heat-coagulated egg-albumen and water therefore followed the partition-law.

In Experiment II, equilibria (A) were obtained by the uptake of phenol by the coagulum, and equilibria (B) by the reverse process. The similarity in the values of the distribution-ratios deduced from the two series of equilibria showed that the absorption was reversible.

The mean partition-coefficient in Experiment I was 9·8, while in Experiment II it was 12·9. The coagula used were prepared from different batches of albumen. This may explain the difference in these values which, however, may also have its explanation in the varying conditions existing during the formation of the heat-coagula.

A comparison of the solvent power of the heat-coagulum for phenol with that of unprecipitated egg-albumen and of the phenol precipitated protein (Tables II and III) indicates that heat-coagulation was accompanied by an increase in the absorptive capacity of this protein for phenol, and that, except in low concentrations, heat- and phenol-coagulation affected the distribution of phenol between egg-albumen and water to somewhat similar degrees. Moore and Bigland[5] showed that the uptake of alkali by egg-albumen was also increased by heat-coagulation.

(*d*) *Heat-coagulated egg-globulin*

TABLE IV

EQUILIBRIA

½ gram coagulum ; 25 c.c. phenol solution

Water-phase			
Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)	Amount of phenol in grams taken up by 1 gram coagulum	Distribution-ratio $\dfrac{\text{Amount of phenol held by 1 gram protein}}{\text{Amount of phenol held by 1 gram water}}$
5·195	4·470	0·524	11·7
4·632	3·871	0·420	10·8
4·572	3·810	0·408	10·7
1·977	1·653	0·162	9·8
0·505	0·416	0·044	10·6
0·373	0·342	0·039	11·4
0·166	0·201	0·027	13·5
0·247	0·199	0·024	12·0
			Mean = 11·3

The distribution of phenol between water and the coagulated globulin therefore followed the partition-law, and phenol was 11·3 times as soluble in the protein as in water. By comparing the above results with those in Table III it is found that the solubilities of phenol in heat-coagulated egg-albumen and globulin were approximately the same.

(*e*) *Casein*

Casein was not precipitated from its solutions in sodium hydroxide by phenol. The experiments described below were carried out with the precipitated casein of commerce. The maximum amount of phenol was absorbed by casein in forty hours, which time was therefore allowed for equilibrium to be established.

Table V

EQUILIBRIA

½ gram casein ; 25 c.c. phenol solution

	Water-phase		Amount of phenol in grams taken up by 1 gram casein (by difference)	Distribution-ratio $\frac{\text{Amount of phenol held by 1 gram protein}}{\text{Amount of phenol held by 1 gram water}}$
	Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)		
Experiment I (Diagram IV)				
1	7·485	5·614	0·936	16·6
2	5·233	4·418	0·662	15·0
3	4·491	3·491	0·500	14·3
4	1·396	1·888	0·254	13·5
5	1·497	1·227	0·135	11·0
6	0·491	0·633	0·064	10·1
7	0·253	0·315	0·033	10·4
8	0·126	0·156	0·018	11·5
Experiment II	0·409	0·344	0·033	9·6
2	1·137	0·986	0·108	11·0
3	3·634	3·125	0·363	11·6
4	4·966	4·508	0·592	13·1
5	5·527	5·266	0·722	13·7

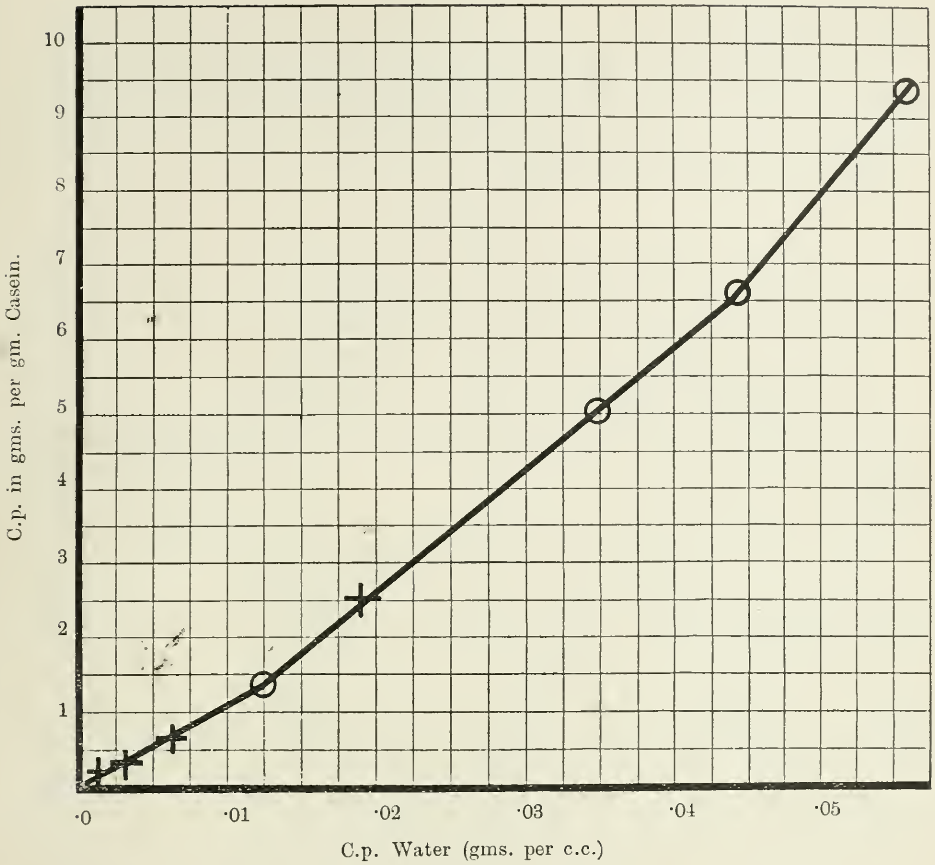

Diagram IV.—Equilibria with phenol between water and casein. (*Experiment I*, Table V.)

O = Equilibria obtained during rising phenol concentration.

+ = Equilibria obtained by reverse process.

The absorptive capacity of casein for phenol was thus considerably greater than that of water, and was of the same order of magnitude as the absorptive capacities of other precipitated proteins for this substance. The results of Experiment I indicated that although below a final phenol concentration of 1·8 per cent., the distribution of phenol between water and casein followed the partition-law, at 1·8 per cent. there was a deviation therefrom, the distribution-ratio increasing with further rise in concentration. The results of Experiment II also indicated this deviation, but there were not sufficient equilibria determined in this experiment to show the constancy in magnitude of the distribution-ratio at low concentrations.

Equilibria 1, 2, 3, 5, in Experiment I were obtained by the uptake of phenol by casein; the remaining equilibra by the reverse process. The coincidence of the two series (Diagram IV) showed that the absorption of phenol by casein was reversible.

II

THE INFLUENCE OF TEMPERATURE UPON THE DISTRIBUTION OF PHENOL BETWEEN WATER AND PROTEINS

(*a*) *Egg-albumen in solution*

In the following table are given the amounts of phenol absorbed by a definite amount of protein from the same concentration at 20° C. and 37·5° C.

TABLE VI.—10 c.c. 11·2 per cent. egg-albumen solution; 50 c.c. phenol solution

	Water-phase			
Temperature	Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)	Amount of phenol in grams taken up by 1·0 gram protein	Distribution-ratio
20° C.	0·248	0·196	0·006	3·0
20° C.	0·248	0·196	0·006	3·0
37·5° C.	0·248	0·197	0·005	2·5

A rise in temperature of 17·5° C. thus had no measurable effect upon the uptake of phenol by unprecipitated egg-albumen.

(*b*) *Heat-coagulated egg-albumen*

The experiments with the heat-coagulum were also carried out at 20° C. and 37·5° C.

TABLE VII

EQUILIBRIA

	Water-phase = 25 c.c.				
Temperature	Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)	Weight of coagulum taken in grams	Amount of phenol in grams taken up by the coagulum	Distribution-ratio
20° C.	0·584	0·515	0·35	0·017	9·7
20° C.	0·984	0·702	1·0	0·070	10·0
20° C.	0·984	0·705	1·0	0·070	9·9
37·5° C.	0·984	0·731	1·0	0·063	8·6
37·5° C.	0·984	0·732	1·0	0·063	8·6
37·5° C.	0·584	0·524	0·35	0·015	8·2

The results show that the uptake of phenol by the coagulum was slightly decreased by a rise in temperature from 20° C. to 37·5° C.

(*c*) *Gelatin*

As gelatin dissolves in water at temperatures above 20° C., experiments were carried out at lower temperatures.

TABLE VIII

EQUILIBRIA

Gelatin, 4 grams; phenol solution, 100 c.c.

Temperature	Water-phase: Initial concentration (grams per 100 c.c.)	Water-phase: Final concentration (grams per 100 c.c.)	Amount of phenol in grams absorbed by protein (4 grams)	Distribution-ratio
4·4° (Minimum) to 6·6° C. (Maximum) = 5·5° C. Mean	0·994	0·914	0·080	2·2
	0·994	0·922	0·072	1·9
20° C.	0·994	0·914	0·080	2·2
20° C.	0·994	0·916	0·078	2·1
20° C.	0·994	0·917	0·077	2·1

A rise in temperature of about 15° C. therefore caused no change in the distribution of phenol between water and gelatin.

The results indicate that, although the solubilities of phenol in water, gelatin and dissolved egg-albumen were increased equally by rise in temperature, the solubility of phenol in heat-coagulated egg-albumen was increased to a slightly smaller extent than that of the same substance in water.

III

THE INFLUENCE OF ALCOHOL UPON THE DISTRIBUTION OF PHENOL BETWEEN WATER AND HEAT-COAGULATED EGG-ALBUMEN

A definite amount of coagulum was added to each of a number of phenol solutions containing different percentages by volume of alcohol. The mixtures were kept at 20° C. for four days, and the filtrates from the coagula then analysed. Alcohol was found not to interfere with the process of estimating phenol. The results are tabulated below.

TABLE IX.—1 gram coagulum ; 100 c.c. phenol solution

Water-phase: Initial concentration (grams per 100 c.c.)	Water-phase: Final concentration (grams per 100 c.c.)	Per cent. of alcohol initially present (by volume)	Amount of phenol in grams taken up by 1 gram of protein (by difference)	Distribution-ratio Amount of phenol held by 1 gram protein / Amount of phenol held by 1 gram water
1·992	1·680	0	0·156	9·3
1·992	1·684	0	0·154	9·2
0·993	0·833	0	0·080	9·6
1·992	1·758	25	0·117	6·7
1·992	1·770	25	0·113	6·4
0·993	0·974	50	0·010	1·0

These experiments indicate that alcohol considerably decreased the uptake of phenol by the protein. The alcohol as well as the phenol would be absorbed by the coagulum and, since alcohol is a very efficient solvent for phenol, its distribution between water and protein would lead to an increased solubility of phenol in both phases. The results of the above experiments therefore mean that the amount of alcohol absorbed was not sufficiently great to increase the solvent power of the protein for phenol to as great an extent as the alcohol unabsorbed increased the solvent power of the water for this substance.

IV

The Influence of Hydrochloric Acid upon the Distribution of Phenol between Water and Heat-Coagulated Egg-Albumen

Hydrochloric acid decreases the solubility of phenol in water, and it was therefore of interest to investigate its influence on the distribution of phenol between water and proteins. Definite amounts of the coagulum were immersed in a number of phenol solutions containing different concentrations of hydrochloric acid. After equilibrium was established, the phenol contents of the filtrates were estimated. The results are tabulated below.

TABLE X.—0·5 gram coagulum ; 25 c.c. phenol solution

Water-phase: Initial concentration (grams per 100 c.c.)	Water-phase: Final concentration (grams per 100 c.c.)	Concentration of acid (HCl)	Amount of phenol in grams taken up by 1 gram coagulum (by difference)	Distribution-ratio Amount of phenol taken up by 1 gram protein / Amount of phenol taken up by 1 gram water
Experiment I				
0·996	0·824	0	0·085	10·3
0·996	0·793	N × 3	0·101	12·7
0·996	0·805	N × 6	0·095	11·8
Experiment II				
0·983	0·808	0	0·087	10·7
0·983	0·784	N × 3	0·100	12·7
0·983	0·797	N × 5	0·093	11·7
0·983	0·801	N × 5	0·091	11·3

The presence of hydrochloric acid therefore slightly increased the uptake of phenol by the coagulum, but the increase was more marked in lower concentrations of acid than in higher ones.

The hydrochloric acid, as well as the phenol, would be absorbed by the protein, and it would seem that the amount of acid absorbed was not sufficient to decrease the solubility of phenol in the protein to as great an extent as the amount of acid left in aqueous solution decreased the solubility of phenol in water.

On comparing the results of the experiments with alcohol and acid with those of Reichel's[1] experiments on the influence of sodium chloride upon the uptake of phenol by proteins (see introduction), it is found that a substance increasing the solubility of phenol in water diminishes the uptake of phenol by a protein, while substances decreasing the solubility have exactly the opposite effect.

V

The Influence of Concentration upon the Distribution of Meta-cresol between Water and Proteins

(*a*) *Gelatin*

Saturated aqueous solutions (1·25 per cent.) of ortho-, meta- and para-cresols had no precipitating action upon gelatin. Below are tabulated some equilibria with meta-cresol between water and gelatin, and also some equilibria with phenol determined under the same conditions.

Table XI.—5 grams gelatin ; 125 c.c. phenol or cresol solution

Water-phase		Amount of phenol or cresol in grams absorbed by 5 grams gelatin (by difference)	Distribution-ratio $\frac{\text{Amount of phenol or cresol absorbed by 1 gram protein}}{\text{Amount of phenol or cresol absorbed by 1 gram water}}$	
Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)			
Experiment I	Meta-cresol			
1·040	0·946	0·140	2·9	Mean =3·0
0·832	0·757	0·122	3·2	
0·624	0·570	0·082	2·8	
Experiment II	Phenol			
0·906	0·840	0·102	2·4	Mean =2·3
0·725	0·672	0·080	2·4	
0·544	0·505	0·057	2·2	

Over the small concentration range possible to work with, owing to the slight solubility of cresol in water, the distribution of meta-cresol between water and gelatin followed the partition-law, and quantitatively was very similar to that of phenol between the same solvents.

Since meta-cresol is much less soluble than phenol in water, the above results indicate that the introduction of a methyl group into the benzene nucleus of phenol decreased its solubilities in water and gelatin to approximately the same degree.

(*b*) *Egg-albumen* (*dialysed solution*)

A 0·7 per cent. solution of meta-cresol was sufficient to produce a turbidity in a 10 per cent. egg-albumen solution, and a 1 per cent. solution immediately produced a copious precipitate of protein, which was irreversible. A 1 per cent. solution of phenol, however, only caused a faint turbidity in an egg-albumen solution of the same strength. The introduction of a methyl group into the benzene ring of phenol therefore increased its precipitating action upon egg-albumen.

In the following table are given some equilibria with meta-cresol between water and egg-albumen.

Table XII.—10 c.c. 10·2 per cent. egg-albumen solution; 50 c.c. cresol solution

Water-phase		Amount of meta-cresol in grams taken up by 1 gram protein	Condition of protein	Distribution-ratio Amount of meta-cresol held by 1 gram protein / Amount of meta-cresol held by 1 gram water
Initial concentration (grams per 100 c.c.)	Final concentration (grams per 100 c.c.)			
0·188	0·180	0·005	Unprecipitated	2·8 } Mean =3·1
0·471	0·445	0·015	Unprecipitated	3·4
0·846	0·696	0·104	Precipitated by cresol	14·9
0·950	0·822	0·179	Precipitated by cresol	21·8

Over the small concentration range possible to work with, the distribution of meta-cresol between water and unprecipitated egg-albumen followed the partition-law, and the cresol was 3·1 times as soluble in the protein as in water.

Experiment III in Table II with phenol was carried out under the same conditions as the above experiment with cresol, and it was found that phenol was 2·9 times as soluble in egg-albumen as in water. Egg-albumen, therefore, absorbed about equal amounts of phenol and cresol from solutions of the same percentage strength, so that the solubilities of phenol in water and egg-albumen were equally decreased by the introduction of a methyl group into its benzene nucleus. Since egg-albumen was precipitated by lower concentrations of cresol than of phenol, the above results indicate that the relative precipitating power of these substances was not entirely determined by their equilibrium positions between water and the protein.

A comparison of the distribution-ratio of meta-cresol between water and egg-albumen with that of the same substance between water and gelatin indicated that the solubilities of cresol in the two proteins were equal. It was found that while egg-albumen was precipitated by dilute solutions of meta-cresol, gelatin was not even affected by saturated solutions (1·25 per cent.). As in the case of phenol, the susceptibilities of these proteins to precipitation by cresol were therefore not entirely determined by their absorptive capacities for this substance.

The precipitation of egg-albumen by cresol was accompanied by a large increase in the absorptive capacity of the protein for this substance. This increased absorption took place at a lower concentration with cresol than with phenol owing to the greater precipitating power of the former substance.

VI

THE SOLUBILITY OF PROTEINS IN PHENOL AND META-CRESOL

Ritthausen (1872),[6] and Osborne[7] and his co-workers (1891-93), found that zein dissolved in warm crystallized phenol. Kjeldahl (1896)[8] showed that gliadin was also soluble in para-cresol and was precipitated from its phenol solution by many organic reagents.

Reichel (1909)[1] found that when serum was warmed with anhydrous phenol a clear solution resulted which underwent no apparent change on boiling.

The high solvent powers of egg-albumen and gelatin for phenol suggested that these proteins would also be soluble in anhydrous phenols.

When egg-albumen crystals were heated with molten phenol at 45° C. the protein gradually dissolved, and a clear solution was obtained. On dialysis or the addition of water to this solution the protein was precipitated. This was also effected by dialysing at 45° C. Similarly heat-coagulated egg-albumen dissolved in molten phenol and was reprecipitated when the solution was diluted. Gelatin dissolved in phenol less readily than egg-albumen. It could be recovered in the unprecipitated state from the solution by dialysis at ordinary temperatures.

Meta-cresol also dissolved egg-albumen and gelatin at temperatures of about 40° C. Solution was not detected at room temperatures. By dialysing these solutions at room temperatures while the egg-albumen separated as a coagulum, the gelatin was recovered in the unprecipitated condition.

The facts that egg-albumen could only be recovered from these solutions in the precipitated state, while gelatin could be recovered in an unaltered condition, corresponded with the known differences in the stability in the presence of water or dilute phenol solutions of the products of the precipitating action of the phenols upon these proteins.

When anhydrous meta-cresol was added to 1/2 its volume of horse-serum at ordinary temperatures the serum proteins were precipitated, but on heating to 100° C. these were redissolved and a clear homogeneous fluid resulted. On cooling the liquid again became turbid owing to separation of the proteins. Meta-cresol rapidly dissolved Witte's peptone at 100° C., forming a brown liquid. Solution was also found to proceed slowly at ordinary temperatures.

Although meta-cresol dissolved proteins readily, it had no solvent action, even at high temperatures, upon the amino acids, glycyl-l-tyrosin and di-alanyl-cystin. These amino-acids also differed from proteins in not being precipitated from their aqueous solutions by strong solutions of phenol.

VII

The Application of Some of these Facts to the Theory of Phenol Disinfection

1.—The influence of alcohol upon the germicidal power of phenol

Kronig and Paul (1897)[10] showed that the germicidal action of phenol upon anthrax spores was decreased by the presence of alcohol, a solution of phenol in 98 per cent. alcohol possessing only a very feeble bactericidal power.

The experiments described in Section III showed that the presence of alcohol decreased the uptake of phenol by heat-coagulated albumen. The decrease was of such magnitude that the protein absorbed eight times as much phenol from an aqueous solution as from a solution of phenol in 50 per cent. alcohol.

The inhibiting influence of alcohol upon the bactericidal action of phenol is sufficiently explained by a decreased uptake of phenol by the spore-proteins, i.e., to a decreased amount of phenol available for disinfection.

It may be pointed out in this connection that, although the addition of acids considerably increases the germicidal action of phenol and cresol, the presence of hydrochloric acid only very slightly increased the uptake of phenol by egg-albumen. This suggests that the effect of acid upon germicidal power is only to a small extent due to an increased uptake of phenol by the bacterial proteins. It is probably due chiefly to the bactericidal action of the acids themselves.

II.—The effect of the presence of organic matter upon the germicidal action of phenol and cresol

Blyth,[11] in 1906, showed that the bactericidal action of phenol and cresol was decreased by the presence of milk, and to a lesser extent by separated milk. As the latter contains the proteins and sugar of the original milk with only a trace of fat and as lactose was found to have practically no effect upon the germicidal action of phenol, the depreciating effect of separated milk upon bactericidal action must be due to its constituent proteins. Some experiments recorded in Section I showed that casein absorbed phenol from aqueous solution. In a mixture of separated milk and phenol solution the casein therefore renders a considerable portion of the germicide unavailable for disinfection, and in this rests the explanation of the depreciating effect of separated milk upon bactericidal action.

III.—The influence of temperature upon the germicidal action of phenol

Henle (1889)[12] showed that the disinfection of *B. typhosus* was more rapidly completed if the temperature of medication were raised.

H. Chick (1908)[13] quantitatively determined the influence of temperature upon the rate of disinfection of *B. paratyphosus* by phenol. It was found that the velocity of disinfection increased seven to eight fold for a rise in temperature of 10° C.

Since it was found that the uptake of phenol by an unprecipitated protein (gelatin) proceeded very rapidly, being complete within two minutes even at low concentrations, the influences of temperature upon the equilibrium positions of phenol between water and proteins and upon the velocity of disinfection can be compared. It was shown that a rise in temperature of about 15° C. had no measurable effect upon the distribution of phenol between water and unprecipitated proteins. This suggests that the great accelerating influence of rise in temperature upon the process of disinfection is not due to an increased uptake of phenol by the bacterial proteins, but must be due to an increased velocity of a reaction between the phenol and proteins which proceeds subsequently to the absorption.

IV.—*The unequal germicidal powers of phenol and meta-cresol*

Several investigators have shown that the germicidal power of meta-cresol exceeds that of phenol. This is indicated in the following table, in which the number of times meta-cresol exceeds phenol in germicidal power is represented by the carbolic acid co-efficient.

Organism	Carbolic acid co-efficient of meta-cresol (Pure phenol = 1·0)
B. typhosus	2·6
Staphylococcus py. aur	2·0
B. coli	2·1

The experiments described in Section V showed that, although meta-cresol was superior to phenol in its precipitating action upon egg-albumen, there was no difference in the amounts absorbed by this protein from equal concentrations of the two substances.

The introduction of a methyl group into the benzene nucleus of phenol therefore led to an increase in both bactericidal power and protein-precipitating power, but to no change in the uptake of the phenol by the protein. This suggests that the superiority of meta-cresol over phenol as a germicidal agent is due to the fact that cresol precipitates proteins in smaller concentrations than phenol.

V.—*Selective germicidal action*

The germicidal power of a substance depends upon the nature of the organism employed. This is indicated in the following table :—

Organism	Concentration of phenol killing in 15 mins. under constant conditions
B. typhosus	7 in 1,000
B. diphtheria	5 in 1,000
B. coli	8 in 1,000
Staphyloccus py. aur	10 in 1,000
B. pestis	7 in 1,000

The cresols exhibit similar cases of selective germicidal action.

Some experiments described in a previous section showed that proteins possessing equal absorptive capacities for a phenol were unequally susceptible to its precipitating action. This suggests that the phenomenon of selective germicidal action is determined by the phenol concentration at which the specific proteins are precipitated.

SUMMARY AND CONCLUSION

I.—On the relations of phenol and meta-cresol to proteins

1. Gelatin and egg-albumen absorb phenol and meta-cresol according to the partition-law.

2. When a certain phenol concentration is reached the proteins are precipitated. This is accompanied by a great increase in their absorptive capacities for phenol.

3. The precipitation of gelatin by phenol is reversible, and the absorption of phenol by this protein is also reversible throughout the whole range of phenol concentration in water. The precipitation of egg-albumen, on the other hand, is irreversible, and the precipitated protein retains permanently its high absorptive capacity for phenol.

4. Heat coagulation also increases the solvent power of egg-albumen for phenol and, except at low concentrations, influences the equilibrium of phenol between water and this protein to about the same degree as phenol precipitation.

5. Meta-cresol does not precipitate gelatin, but it precipitates egg-albumen at lower concentrations than phenol. The precipitation leads to an increase in the absorptive capacity of the protein for cresol.

6. The solubilities of phenol in gelatin and egg-albumen at 20° C. are equal. This is also true of meta-cresol. Although this is the case, egg-albumen is precipitated by lower phenol and cresol concentrations than gelatin. The susceptibilities of the two proteins to precipitation are therefore not entirely determined by their absorptive capacities for the phenols.

7. Egg-albumen absorbs identical amounts of phenol and meta-cresol from equal percentage concentrations, but is precipitated by lower concentrations of cresol than of phenol. The relative precipitating power of these substances is therefore not entirely determined by their equilibrium positions between water and the protein.

8. Heat-coagulated egg-albumen and egg-globulin absorb phenol according to the partition-law. Their solvent powers for phenol are approximately equal.

9. Casein at low concentrations absorbs phenol according to the partition-law. Above 1·8 per cent., however, with rising concentration there is an increased proportional uptake of phenol. The absorption is reversible. Casein possesses the high absorptive capacity of precipitated proteins.

10. Rise in temperature has no measurable effect upon the distribution of phenol between water and egg-albumen and gelatin; it leads, however, to a slightly decreased uptake of phenol by heat-coagulated egg-albumen.

11. Alcohol considerably decreases the uptake of phenol by heat-coagulated egg-albumen. Hydrochloric acid, on the other hand, slightly increases the uptake.

12. Egg-albumen (crystallized or heat-coagulated) and gelatin dissolve in warm anhydrous phenol and meta-cresol. When the solutions are dialysed, the albumen is irreversibly precipitated, while the gelatin can be recovered in the unaltered condition.

13. Horse-serum forms a homogeneous fluid with about twice its volume of meta-cresol at 100° C. On cooling the serum proteins are precipitated.

14. Although meta-cresol dissolves proteins and also peptone, it does not dissolve even at high temperatures the polypeptides—glycyl-l-tyrosin and di-alanyl-cystin. These substances are also not precipitated from aqueous solution by strong phenol solutions.

II.—The application of some of these facts to the theory of disinfection

1. The depreciating effect of alcohol upon the bactericidal action of phenol is explained by a reduction in the absorptive capacities of the bacterial proteins for this substance. The action of hydrochloric acid in increasing the germicidal power of phenol, on the other hand, is only partially explained by a redistribution of phenol between water and proteins.

2. The depressing effect of separated milk upon the germicidal action of phenol is due to the absorptive capacity of the constituent casein for this substance.

3. The great increase in the germicidal power of phenol, which results from rise in temperature, is not explained by the influence of temperature upon the distribution of phenol between water and proteins.

4. The superiority of meta-cresol to phenol as a germicide appears to be due to the fact that cresol precipitates proteins in lower concentrations than phenol.

5. The selective action of phenol as a germicide upon different organisms seems to be associated with the observed dissimilar susceptibilities of proteins to its precipitating action.

Main Conclusion

The absorption of the phenols by bacteria is merely the initial stage in the process of disinfection.

The germicidal action which follows the absorption does not seem to be the result of a typical chemical union between the phenols and bacterial proteins, as is the case, for instance, with formaldehyde, but is apparently associated with the de-emulsification of the colloidal suspension as evidenced by the precipitation of proteins when a certain phenol concentration is attained. In the case of egg-albumen the change is irreversible, and the precipitated protein is not again dispersed on the removal of the phenol. The germicidal action of phenol thus appears to be similar in mechanism to that of heat. This interpretation of the process of disinfection by phenol may explain the fact that below certain concentrations (about 0·5 per cent.) this substance exerts but a very feeble bactericidal action, there being a disproportionate falling off in germicidal power when the phenol concentration is reduced to this point.

I desire to express my best thanks to Dr. C. J. Martin, F.R.S., for much helpful advice in the course of this work.

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XVI. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART II. A COMPARATIVE STUDY OF THE EFFECTS OF VARIOUS FACTORS UPON THE GERMICIDAL AND PROTEIN-PRECIPITATING POWERS OF THE PHENOLS.

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(*Received January 13th, 1913.*)

Evidence set forth in a previous communication on this subject [Cooper, 1912, 2] suggested that the germicidal power of the phenols is due, not to a typical chemical action upon the bacterial proteins, as appears to be the case with formaldehyde, but to a de-emulsifying effect upon their colloidal suspensions as evidenced by the precipitation of proteins when a certain phenol concentration is attained.

Since H. Chick [1910] has shown that disinfection by heat is analogous to the heat-coagulation of proteins, it seems that a similarity in mechanism exists between the germicidal actions of heat and of the phenols.

Hardy [1899] pointed out that the heat-coagulation of proteins consists of two processes, namely, (1) The reaction between the protein and hot water (denaturation). (2) The subsequent separation of the altered protein in a particulate form (agglutination). It is not at present known whether the essential stage in the processes of disinfection by heat and the phenols is a denaturation of the bacterial proteins or a destruction of their colloidal suspension.

The experimental work described in this paper has been undertaken with the object of studying more closely the apparent relationship between the bactericidal action of the phenols and their precipitating effects on proteins.

I. The Influence of Chemical Constitution upon the Germicidal and Protein-Precipitating Action of the Phenols.

The experimental method. The minimum concentrations in water of various phenols and certain other substances have been determined at which a visible change in the state of aggregation of the protein occurred. Small alterations in the magnitude of the protein-particles brought about by such concentrations were indicated by a turbidity; larger alterations by a progressively increasing precipitation of the colloid as the concentrations of the phenols rose.

Two proteins were used in the experiments, namely, gelatin and dialysed egg-albumin; the former was employed in the form of small strips and the latter as its colloidal suspension in water. The effect on the gelatin was to produce a turbidity in the strip immersed in the solution under examination. Throughout the experiments the weight of protein, volume of solution, and temperature (20°) were kept constant. Observations of the effects of the various solutions upon the proteins were made immediately after admixture and also after standing one hour. The results tabulated below are based on the observations made after the one hour's standing.

The experimental results. The protein-precipitating powers of the various substances have been compared with their germicidal activities, which are expressed in the table below in terms of the concentrations required to disinfect *B. typhosus* or *Staphylococcus pyogenes aureus* in 15 minutes under the standard conditions employed by Chick and Martin [1908]. The concentrations have been selected from two previous papers on disinfection (1) by Morgan and Cooper [1912] and (2) by the author [1912, 1], to which papers the numbers 1 and 2 in the following table (p. 177) refer.

The following parallelisms can be deduced from these results.

1. The germicidal and protein-precipitating powers of phenol are decreased by the introduction of hydroxyl-groups into its benzene nucleus and increased by the introduction of nitro-groups and also by that of a methyl group.

2. The monohydric phenols are superior to the monohydric alcohols and also to acetone both as germicides and protein-precipitants.

3. The effects of substitution in the molecule of phenol upon its germicidal and protein-precipitating powers are frequently of somewhat similar orders of magnitude. An exact correspondence was not expected, firstly, because it is well known that the carbolic acid coefficient of a

substance varies to some extent with the species of micro-organism employed, and, secondly, because bacterial proteins were not used in the determination of protein-precipitating power.

TABLE I.

Substance	*Protein-precipitating power* Minimum concns. at which precipitation was visible. (Percentages in terms of weight per volume)	*Germicidal power* Concentrations killing in 15 minutes. (Percentages in terms of weight per volume)	*Organism*
	Gelatin 0·1 g.	Vol. of liquid 10 c.c.	
Phenol ...	2·5	0·8 [1]	*B. typhosus.*
Trinitrophenol (Picric acid)	0·2	0·1 [1]	,,
Resorcinol ...	4·0	2·6 [2]	,,
Catechol ...	5·0	1·7 [2]	,,
Quinol ...	5·0	0·75 [2]	,,
Pyrogallol ...	3·0	1·0 [2]	,,
Phloroglucinol	Satd. solns. (5$^0/_0$) had no visible effect	>2·3 [2]	,,
p-Nitrophenol	0·9	0·46 [1]	*Staphylococcus py. aur.*
m-Nitrophenol	0·6	0·27 [1]	,, ,,
Trinitrophenol (Picric acid)	0·2	0·14 [1]	,, ,,
Ethyl alcohol	50 $^0/_0$ and 100 $^0/_0$ had no visible effect	35·0 [1]	,, ,,
Acetone		>10·6 [2]	,, ,,
Phenol ...	2·5	1·0 [1]	,, ,,
	Egg-albumin ½ c.c. 10 $^0/_0$ solution (0·05 g.).	Vol. of solution containing precipitant = 4·5 c.c.	
Phenol ...	1·0	0·8 [1] 1·0 [1]	*B. typhosus.* *Staphylococcus py. aur.*
m-Cresol ...	0·7	0·5 [2] 0·3 [2]	,, ,, *B. typhosus.*
Acetone ...	12·0	>10·6 [2]	*Staphylococcus py. aur.*
Methyl alcohol	20·0	35·0 [1]	,, ,,
Ethyl alcohol	20·0	35·0 [1] 32·5 [1]	,, ,, *B. typhosus.*
Propyl alcohol	12·0	14·0 [1]	*Staphylococcus py. aur.*

II. THE EFFECTS OF VARIOUS SUBSTANCES UPON THE GERMICIDAL AND PROTEIN-PRECIPITATING ACTION OF THE PHENOLS.

The experimental method employed in the investigation of the precipitating action of the phenols was similar to that described in Section I. The various substances were added to the phenol solutions immediately before admixture with the protein. The results recorded in this section are based on observations of the condition of the proteins after one hour's contact with the phenol solutions.

A. Sodium Chloride.

Reichel [1909] showed that the addition of sodium chloride increased both the bactericidal action of phenol and its solubility in proteins. The germicidal power was about doubled by 10 per cent. salt and quadrupled by 20 per cent. salt, and the effects of salt upon the solubility in proteins were of similar orders of magnitude. Smaller amounts of salt had little influence.

These experiments indicated how in the presence of salt a larger amount of phenol became available for disinfection, but did not throw any light on the mechanism of the germicidal action of this disinfectant. It was therefore of interest to investigate the influence of salt upon the protein-precipitating action of phenol.

Gelatin. The minimum concentrations of phenol, which induced a visible precipitation in the presence of varying amounts of sodium chloride, were determined. The results are tabulated below:

TABLE II.

Percentage of salt present (weight per volume)	Minimum phenol concentrations precipitating the protein
0	3 %
5	2·5
12	1·5
19	0·75

Although saturated aqueous solutions of *m*-cresol (1·25 per cent.) had no visible effects upon gelatin, this protein was precipitated by 0·8 per cent. solutions of *m*-cresol in the presence of 9 per cent. salt and even by 0·4 per cent. solutions in the presence of 20 per cent. of salt.

Dialysed egg-albumin. 3·3 per cent. aqueous solutions of phenol only induced a faint turbidity in a sample of egg-albumin, but in the presence of 6 per cent. salt an extensive precipitation occurred. 1 per cent. solutions of phenol in water and in 6 per cent. salt had no visible effect on the albumin suspension; a turbidity however was induced by the same phenol concentration in 15 per cent. salt, and a marked precipitation in 22 per cent. salt solution.

The precipitating action of phenol upon proteins was thus greatly increased by the presence of salt, but was not appreciably affected until high salt concentrations were attained.

A comparison of the results of experiments with gelatin with those obtained by Reichel [1909] indicates that the germicidal power, the solubility in proteins, and the precipitating action of phenol are increased to very similar extents by the presence of salt.

The increased germicidal power thus appears to be due to an increased de-emulsifying action of the phenol upon the bacterial proteins resulting from the availability within the emulsoid particles of a larger amount of phenol.

B. ALCOHOL.

Kronig and Paul [1897] showed that the germicidal action of phenol upon spores was decreased by the presence of alcohol and that solutions of phenol in 98 per cent. alcohol possessed no bactericidal power.

The author [1912, 2] found that the uptake of phenol by a protein was greatly decreased by the presence of alcohol, the protein absorbing for example eight times as much phenol from aqueous solution as from a solution of phenol in 50 per cent. alcohol. These experiments indicated how in the presence of alcohol less phenol became available for disinfection, but they afforded no explanation of the nature of its germicidal action. It was therefore of some importance to investigate the influence of alcohol upon the protein-precipitating action of phenol.

Gelatin in the form of strips was chiefly employed in the experiments, because it was not precipitated by immersion in alcohol of any strength. Although this protein was precipitated by 2½ per cent. aqueous phenol solutions, there was no indication of precipitation by 5 per cent. phenol solutions in 50 per cent. alcohol, and the extent of the precipitation by 6·25 per cent. phenol solutions in 25 per cent. alcohol was much less than that induced by the above aqueous solutions. Furthermore, 10 per cent. solutions of phenol in absolute alcohol had no visible effect upon gelatin. This is of interest when correlated with Kronig and Paul's observation that solutions of phenol in 98 per cent. alcohol possessed no germicidal power.

A sample of dialysed egg-albumin was immediately precipitated by 1·5 per cent. aqueous phenol solutions, but was not visibly affected by the same concentration in the presence of 10 per cent. alcohol.

Although 0·5 per cent. aqueous solutions of picric acid precipitated gelatin, similar concentrations in 30 per cent. alcohol had no visible effect upon this protein.

The inhibiting effect of alcohol upon the germicidal power of phenol thus appears to be due to a decrease in the de-emulsifying action of the latter upon the bacterial proteins, resulting from a diminution in the amount of phenol available within the emulsoid particles.

C. FAT.

Strips of gelatin were immersed in castor-oil, containing varying amounts of phenol. It was found that solutions of phenol in the fat as strong as 10 per cent. (by weight) had no visible precipitating action upon gelatin, although this protein was precipitated by 2½ per cent. aqueous solutions of phenol.

This can be correlated with the fact discovered by Koch that solutions of carbolic acid in oil possess no germicidal power. The greatly diminished protein-precipitating action of phenol in the presence of fat is probably due, as in the case of alcohol, to a decreased uptake of phenol by the protein, because, while phenol is about three times as soluble in gelatin as in water, the partition-coefficient for phenol between fat and water is six.

D. ALKALI.

Kronig and Paul [1897] showed that, while 4 per cent. solutions of phenol in water exerted a strong germicidal action upon anthrax spores, solutions of sodium phenate equivalent to 4 per cent. phenol possessed no germicidal power.

Morgan and Cooper [1912] showed that *p*-nitrophenol was about five times as powerful a germicide as its potassium salt. The inhibiting action of alkali upon the agglutination of proteins by heat suggested that the decreased germicidal power might be due to the protective effect upon the bacterial protoplasm of the alkali, produced in the hydrolysis of the alkali phenates, against the precipitating action of the phenols simultaneously liberated. Accordingly the effect of alkali on the protein-precipitating action of the phenols has been studied.

Dialysed serum-albumin: Phenol. The albumin was not visibly affected by 0·9 per cent. solutions of phenol, but was precipitated by 1·7 per cent. phenol. The precipitation was not only completely inhibited by N/50 to N/150 concentrations of caustic soda, but even by a concentration as low as N/300.

Gelatin: Phenol. Although gelatin was precipitated by 2·5 per cent. aqueous phenol solutions, this protein was not visibly affected by 4 per cent. phenol in the presence of N/25 caustic soda and was only very slightly precipitated by the same concentration in presence of N/50 caustic soda.

Traces of organic bases, such as ethylamine and ethylenediamine, also inhibited the precipitation of gelatin and egg-albumin by phenol.

Gelatin: p-Nitrophenol. Gelatin was precipitated by 1 per cent. aqueous solutions of *p*-nitrophenol, but was not visibly affected by the same concentration in the presence of N/100 caustic soda, nor even by 1·4 per cent. (N/10) solutions containing N/10 caustic soda.

Not only could alkali inhibit the precipitation of proteins by the phenols, but when present in concentrations varying from N/1 to N/10 it could disperse egg-albumin already precipitated by phenol.

The results indicate that very low concentrations of alkali are sufficient to inhibit the precipitation of proteins by phenol. As alkali combines with the phenols forming salts, which are partially hydrolysed in solution, the inhibiting concentrations must have been even smaller than those stated above.

In view of these results it was of great interest to investigate the influence of alkali upon the germicidal action of phenol in greater detail, and in particular to study the effects of very low alkali concentrations. The experiments consisted in the estimation of the minimum concentrations of phenol required to disinfect in a specified time under standard conditions in the presence of varying amounts of alkali.

The following results were obtained:

TABLE III.

Organism, B. typhosus.

Concentration of caustic soda present	Minimum concentration of phenol required to disinfect in 15 mins. at 20°
0	9·5 in 1000 (N/10)
N/250	,, ,,
N/50	,, ,,
N/25	11 in 1000 (N/8·5)
N/20	,, ,,
N/15	13·5 in 1000 (N/7)

The germicidal powers of the above concentrations of alkali in the absence of phenol were next determined and are compared in the following table with those of solutions containing initially the same alkali concentrations with varying amounts of phenol.

TABLE IV.

Concentration of NaOH	Time required for complete disinfection
N/500 NaOH	> 5½ hours
N/250 ,,	> 5½ ,,
N/100 ,,	45 minutes
N/50 ,,	20 ,,
N/25 ,,	< 3 ,,
N/15 ,,	< 3 ,,
N/10 ,,	< 3 ,,
N/25 ,, +N/10 phenol	>15 ,,
N/25 ,, +N/8·5 ,,	15 ,,
N/20 ,, +N/10 ,,	>15 ,,
N/20 ,, +N/8·5 ,,	15 ,,
N/15 ,, +N/10 ,,	>15 ,,
N/15 ,, +N/8·5 ,,	>15 ,,
N/15 ,, +N/7 ,,	15 ,,
N/10 ,, +N/12 ,,	<15 ,,
N/10 ,, +N/10 ,,	15 ,,
N/10 ,, +N/9 ,,	>15 ,,
N/10 ,, +N/7 ,,	>15 ,,

The above results indicate that alkali decreased the germicidal power of phenol when present in moderately low concentration. While, however, the precipitation of serum-albumin by phenol was inhibited by an initial concentration of N/300 caustic soda and that of gelatin by N/50 caustic soda, bactericidal power was not measurably affected until the initial alkali concentration was raised to N/25. The precipitating action of phenol was therefore much more sensitive to the inhibiting effect of alkali than its germicidal action. This discrepancy is not sufficiently explained by the slight acidity of the broth culture, nor by the toxic action of the alkali, since an analysis of the results tabulated above indicates that not only did small concentrations of alkali such as N/250 exert a very feeble germicidal action, but furthermore the bactericidal action of the alkali was greatly decreased by the presence of phenol. Owing to this depreciating effect N/10 solutions of alkali, containing initially N/7 or N/9 concentrations of phenol, possessed a smaller germicidal power than those which initially contained lower phenol concentrations such as N/10 and N/12.

It is possible that a larger amount of alkali is required to preserve the colloidal suspension from the action of the phenol in the case of the bacterial proteins than in the cases of gelatin and albumin.

E. ACIDS.

Hailer [1910] showed that acids, arranged according to their germicidal power and also according to their efficiencies in increasing the bactericidal power of phenols, were not in the same order. Thus sulphuric acid exceeded oxalic acid in bactericidal power, but was less effective in increasing the germicidal power of phenol. Some of the experimental results described in the previous pages suggested that the discrepancy might be due to differences in the effects of the acids upon the protein-precipitating action of phenol. This, however, seems not to be the case because it was found that, although acids greatly increased the precipitating action of phenol, sulphuric acid was effective in much lower concentration than oxalic acid.

The effects of alcohol and salt upon the precipitating action were shown to be due to a redistribution of phenol between the water and proteins. The greatly increased precipitating action in the presence of acids however is not sufficiently explained in this way, as it was previously shown [Cooper, 1912, 2] that the addition of acid only very slightly increased the solubility of phenol in protein. It is probably due to the formation of an acid salt of the protein, which is more readily precipitated than the protein itself.

DISCUSSION OF RESULTS.

The parallelisms deduced from the study of the effects of changes in the chemical constitution of phenol and of the presence of certain substances (e.g. salt, alcohol, fat) upon its germicidal and protein-precipitating powers support the conclusion arrived at in the previous communication, namely, that the germicidal action of phenol is associated with an alteration in the condition of the colloidal suspensions of the bacterial proteins as evidenced by the precipitation of such proteins as gelatin and albumin when certain phenol concentrations are attained.

It has already been pointed out that the heat coagulation of proteins consists of two stages.

1. A reaction between the proteins and hot water (denaturation).
2. A separation of the protein in a particulate form (agglutination).

It is therefore of interest to attempt to characterise the essential process in disinfection by the phenols as either a denaturation of the bacterial proteins or a de-emulsification of their colloidal solution. Chick and Martin [1911; 1912] have shown that the process of denaturation by heat is accelerated by acid and alkali, and retarded by salt, while the subsequent process of agglutination is facilitated by low concentrations of acid and salt, but inhibited by high concentrations and also by alkali.

It was shown in Section II of this paper that the bactericidal power of phenol, its protein-precipitating action and solubility in proteins were increased to equal extents by the addition of salt. The latter thus appears to have no *direct* effect upon the germicidal and precipitating actions of phenol, but to increase them merely by raising the phenol concentration within the emulsoid protein-particles. It is therefore not possible by a study of the effects of salt to correlate these properties of phenol with either the denaturating or agglutinating action of heat upon proteins.

The fact, however, that the germicidal action of phenol is inhibited by low concentrations of alkali, although to a less extent than its precipitating action upon proteins, suggests that the essential process in disinfection by phenol resembles the final phase in the heat-coagulation of proteins and consists in the destruction of the colloidal suspensions of the bacterial proteins.

Since the process of disinfection by hot water was found [Chick, 1910] to be accelerated by alkali as well as by acid, it is possible that the essential stage in this process is the denaturation of the bacterial proteins by the action of the water and not the subsequent precipitation. A study of the effects of salt upon the velocity of disinfection by hot water will throw light on this problem.

SUMMARY.

1. The germicidal and protein-precipitating powers of phenol are similarly affected by the entrance of various chemical groups into its molecule.

(*a*) The introduction of hydroxyl groups decreases and that of nitro-groups and of a methyl group increases the bactericidal and protein-precipitating powers of phenol.

(*b*) The monohydric phenols are superior to the alcohols both as germicides and protein-precipitants.

2. Sodium chloride increases the germicidal and protein-precipitating action of phenol through increasing its solubility in proteins.

3. Alcohol, on the other hand, decreases the germicidal and protein-precipitating action of phenol through diminishing its solubility in proteins. Solutions of phenol in absolute alcohol exert no germicidal action upon spores and have no visible precipitating action on gelatin.

4. Solutions of phenol in fat also possess no germicidal power and do not visibly precipitate gelatin.

5. While the presence of very small amounts of alkali is sufficient to inhibit the precipitating action of phenol upon proteins, only moderately low alkali concentrations can effect a measurable decrease in its germicidal power. The explanation of this discrepancy is not apparent.

6. The precipitating action of phenol is increased by the addition of acids.

MAIN CONCLUSION.

From the evidence set forth in the previous communication and in the present paper it is concluded that the absorption of phenols by bacteria is merely the initial stage in the process of disinfection, and that the germicidal action which follows is due, not to a typical chemical union of the phenols with the bacterial protoplasm, as appears to be the case with formaldehyde, but to a de-emulsifying action upon the colloidal suspension of some constituent protein or proteins essential for the vitality of the organisms.

I desire to express my best thanks to Prof. Martin, F.R.S., for much helpful criticism of this work.

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XVII. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART III. THE CHEMICAL ACTION OF QUINONE UPON PROTEINS.

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Blyth and Goodban [1907] found that when pure cresylic acid was exposed to light and air until it had become brown its germicidal power was measurably increased. This was probably due to the formation of derivatives of quinone through the aerial oxidation of the cresols.

Thalhimer and Palmer [1911] however could not detect any increase in germicidal power when phenol was exposed to light and air for some time.

Morgan and Cooper [1912] showed that some of the aromatic amines possessed a higher germicidal power when coloured by long standing than when purified by redistillation. It is probable that the increased germicidal power was due to the production of coloured quinone derivatives, which are known to be formed in the oxidation of aromatic amines.

Thalhimer and Palmer [1911] showed that quinone itself was a very efficient disinfectant and was superior to phenol, cresol, quinol, phenoquinone, and formalin in germicidal power.

The author [1912, 1] confirmed some of the latter results and showed further that quinone was more efficient as a germicide than the aliphatic ketone, acetone.

In previous communications [Cooper, 1912, 2; 1913] evidence was set forth which strongly suggested that the germicidal action of the phenols was due, not to a typical chemical union with the bacterial proteins, but to a de-emulsifying effect upon their colloidal suspensions. In view of the high bactericidal power of quinone it was of great interest to investigate the relations of this ketone to proteins, in order to compare the mechanism of its germicidal action with that of the phenols and to arrive at a conclusion as to the possibility of a relationship between its germicidal efficiency and chemical reactivity.

Würster [1889] showed that when quinone was added to warm solutions of various amino-acids a red coloration was developed.

Raciborski [1907] showed that quinone gave a red coloration not only with amino-acids, but also with peptone and several proteins (egg-albumin, serum-albumin, fibrin, globulin, legumin, and nuclein). Toluquinone reacted similarly to quinone, and xyloquinone also gave a colour-reaction with proteins and peptone, but not with glycine and alanine. Phenanthraquinone and anthraquinone, on the other hand, gave no colour reactions with proteins. The authors put forward no explanation of the above phenomena.

THE EXPERIMENTAL RESULTS.

The investigation described in this paper is divided into three parts.

Part I deals with the relations of quinone to various proteins.

Part II deals with the nature of the chemical action, which quinone was found in Part I to exert upon proteins.

Part III deals with the relation of the chemical reactivity of quinone to its germicidal power.

I. *The relations of quinone to various proteins.*

Gelatin.

When strips of gelatin were immersed in aqueous solutions of quinone (0·1 per cent.) the protein rapidly developed an intense red colour, but retained its transparency. The reaction was irreversible, as the colour was not removed by prolonged boiling with water or absolute alcohol. The red colour was changed to green by the addition of alkali, but was restored by acidification. The altered gelatin was, furthermore, completely insoluble in hot water, and after immersion for 16 hours in quinone solutions the protein was no longer rendered opaque (precipitated) by phenol. Immersion for 30 minutes did not inhibit the precipitation.

Gelatin after immersion in 40 per cent. formalin for 12 hours was also insoluble in hot water and was no longer precipitated by 5 per cent. phenol, although the original gelatin was visibly affected by 2·5 per cent. phenol solutions.

These facts suggest that the quinone was not merely dissolved by the gelatin, but had reacted chemically with the protein. The colour-reaction did not occur when the gelatin was immersed in solutions of quinone in absolute alcohol, and it only took place to a very small extent in 20 per cent. alcohol. Solutions of quinone in toluene also gave no coloration with gelatin.

Similarly, although Witte's peptone was coloured intensely red when suspended or dissolved in aqueous quinone solutions, no coloration was observed when it was suspended in an alcoholic solution of this substance.

The interpretation of these facts is probably that the quinone is *dissolved* in the gelatin and proteoses before the chemical reaction, so that an efficient external solvent for quinone (such as alcohol) by decreasing the uptake of this substance by the colloids can inhibit the colour-reaction. (See the effect of alcohol upon the distribution of phenol between water and proteins, Cooper [1912, 2].)

Alizarin, like anthraquinone, gave no coloration with proteins. When however gelatin was immersed in strong aqueous solutions of sodium alizarin-sulphonate (Alizarin red, $C_{14}H_5O_2(OH)_2SO_3Na$) it assumed an intense red colour, but, unlike gelatin treated with quinone, it was still readily soluble in hot water and precipitated by 5 per cent. phenol, and the colour was rapidly removed by washing with cold water.

Alizarin and its sulphonic derivative thus differed from quinone in not reacting with proteins, and this inactivity may explain the fact that a saturated solution (0·2 per cent.) of alizarin and a 1 per cent. solution of alizarin-red exerted no measurable germicidal action upon Staphylococcus py. aur.

Caseinogen.

(Merck's Casein—prepared according to Hammarsten.)

When immersed in aqueous solutions of quinone this protein assumed a purple colour, which was not removed by washing with water or boiling alcohol. Unlike the original caseinogen the coloured product (after being washed with water only) was insoluble in N/5 soda. Contact with the alkali, however, turned it green, but the purple colour was restored by acidification. The altered caseinogen was very slowly soluble in hot concentrated hydrochloric acid yielding a brown solution, and thus again differed from the original protein, which quickly dissolved in this acid forming a violet solution.

Egg-albumin.

As stated by Raciborski [1907] dialysed egg-albumin when mixed with aqueous solutions of quinone soon gave an intense red coloration. The protein was still coagulated by heat and alcohol, red coagula being formed, which were not decolorised by prolonged washing with water and alcohol. The protein was also precipitated from the red solution by saturated ammonium sulphate. The precipitate was a red flocculent substance, readily soluble in water and becoming dark brown on standing. After this colour change the protein was frequently found to have become almost insoluble in water. The coagula obtained from the original red solution by means of heat, alcohol, and phenol remained permanently red.

Horse-Serum.

When aqueous solutions of quinone were added to horse-serum a red coloration rapidly developed. The serum-proteins were still coagulated by heat and precipitated by alcohol at first reversibly and finally irreversibly. The coagula were red and could not be decolorised by prolonged washing with alcohol or water.

By half-saturation of the red solution with ammonium sulphate a red flocculent precipitate was obtained, which, like the egg-albumin precipitated by ammonium sulphate from solutions containing quinone, became dark brown on standing. The precipitate with magnesium sulphate and the alcohol coagulum, on the other hand, resembled the egg-albumin coagula in remaining permanently red, but they soon became brown when immersed in a solution containing ammonium sulphate and quinone. The cause of these colour-changes could not be discovered.

By acidifying with acetic acid the filtrate from the precipitation with half-saturated ammonium sulphate of the serum containing quinone another red precipitate was obtained, corresponding to the albumin fraction from normal serum. The precipitate quickly redissolved on the addition of water.

It was not found possible to crystallize the quinone derivates of serum and egg-albumin by the application of the usual methods.

Witte's Peptone.

The observation of Raciborski [1907] that solutions of Witte's peptone gave a red coloration with quinone was confirmed. Experiments were next carried out with a view to the isolation of the coloured products.

Witte's peptone has been separated into five constituents by the method of fractional precipitation with alcohol and salt described by Haslam. The following fractions of proteoses have been isolated in this way:

1. Insoluble in 50 per cent. alcohol and water. Crude hetero-proteose.
2. Insoluble in 50 per cent. alcohol and soluble in water.

(1) α-proto-proteoses—precipitated by half-saturated $(NH_4)_2SO_4$.

(2) α-deutero-proteoses—precipitated by saturated $(NH_4)_2SO_4$.

3. Soluble in 50 per cent. alcohol and soluble in water.

(1) β-proto-proteoses—precipitated by half-saturated $(NH_4)_2SO_4$.

(2) β-deutero-proteoses—precipitated by saturated $(NH_4)_2SO_4$.

By similarly fractionating the coloured liquid obtained by mixing solutions of quinone and Witte's peptone together it was possible to isolate corresponding precipitates all of which were highly coloured. Some of these products, however, did not differ merely in colour from the fractionated proteoses. Thus, while the alcoholic precipitate (α-proteoses and hetero-proteoses) obtained from an aqueous solution of Witte's peptone was readily soluble in warm anhydrous *m*-cresol, the corresponding fraction obtained from the aqueous solution after treatment with quinone was only slightly soluble.

Again, the α-proteoses after reacting with quinone were no longer precipitated by formaldehyde, although they were still precipitated by alcohol, mercuric chloride, and phosphotungstic acid. The significance of this difference is discussed later. The fractions obtained from Witte's peptone after treatment with quinone corresponding to the α- and β- protèoses gave the characteristic test for proteoses, namely a precipitate with nitric acid, soluble on warming and reappearing on cooling.

The main conclusion to be drawn from these results is that proteins isolated after treatment with quinone are permanently coloured and frequently changed in solubility and precipitability, and thus appear to be chemically altered. In the following pages the nature of this chemical change and the possibility of its relationship to the toxic action of quinone upon bacteria are discussed.

II. *The nature of the chemical action of quinone upon proteins.*

(i) *The colour reactions given by quinone with simple amines.*

A large number of amines readily gave colorations with quinone in aqueous solution at ordinary temperatures.

(ii) *The colour-reactions with imino-compounds.*

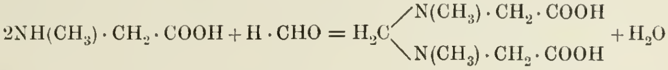
In cold aqueous solution quinone gave red colorations with methylaniline and di-amylamine, but not even on warming with succinimide, acetanilide, and uric acid. Under certain conditions quinone could thus react with substances containing the $=NH$ group.

(iii) *The inhibitory effect of formaldehyde upon the colour-reactions.*

The fact that quinone gives colorations with amino- and imino- compounds suggests that the colour-reaction with proteins is due to a condensation of the ketonic groups of the quinone with their $-NH_2$ and $=NH$ groups.

Formaldehyde is known to react with amino- and imino- compounds by condensation with the $-NH_2$ and $=NH$ groups, forming methylene derivatives.

$$NH_2\cdot CH_2\cdot COOH + H\cdot CHO = CH_2 : N\cdot CH_2\cdot COOH + H_2O$$



$$2NH(CH_3)\cdot CH_2\cdot COOH + H\cdot CHO = H_2C\begin{cases} N(CH_3)\cdot CH_2\cdot COOH \\ N(CH_3)\cdot CH_2\cdot COOH \end{cases} + H_2O$$

The colour reactions given by quinone with proteins, if due entirely to a similar condensation, should therefore be inhibited by the previous formalisation of these substances.

Substance	Nature of reaction
Ammonia	Brown coloration.
Ammonium sulphate ...	Purple ,,
Ethylamine	Violet ,,
Di-amylamine	Rose red ,,
Guanidine (carbonate) ...	Intense green coloration. (Hot solutions brown.)
Glucosamine	Red coloration.
Amino-antipyrine	Purple ,,
Tryptophane	Red ,,
Atoxyl	Red ,,
Aniline	Red ,, (followed by brown precipitate).
o-Phenylene-diamine ...	
m-Tolylene-diamine ...	Violet ,, ,, ,, ,,
β-Naphthylamine	Brown ,,
Tetra-hydro-β-naphthylamine	Brown oil.
Pyridine	Dark yellow coloration passing to red.
Succinanilamine	Red coloration (followed by red precipitate).

It was actually found that in the case of egg-albumin, serum-proteins proteoses glycocoll, glycyl-*l*-tyrosine, methylaniline, and di-amylamine the colour reactions with quinone were entirely inhibited by adding 40 per cent. formalin to the compounds either previously to or simultaneously with the addition of the quinone. Smaller amounts of formaldehyde (one to ten per cent.) added with the quinone were found to inhibit the colour-reaction partially.

Conversely, the *a*-proteoses present in Witte's peptone after treatment with quinone were no longer precipitated by formaldehyde, although they were still precipitable by alcohol, phosphotungstic acid, and mercuric chloride.

In the case of gelatin, however, formalisation did not inhibit the colour-reaction with quinone. This was also true in the case of ammonia, and here the absence of any inhibiting effect could not be due to incomplete interaction with formaldehyde, because the product of this reaction—hexamethylenetetramine (urotropin), which was proved to be ammonia-free, readily gave a red coloration with quinone.

The purified product from the interaction of formaldehyde and aniline also gave a colour-reaction with quinone.

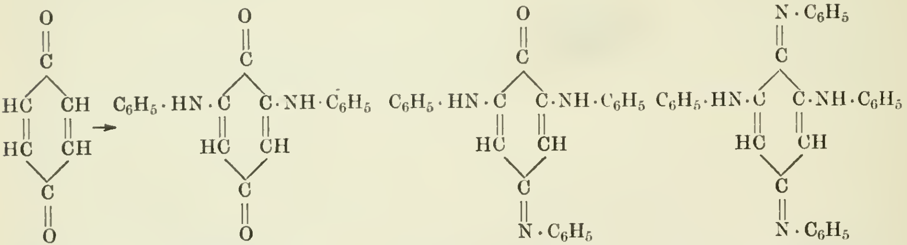
The inhibiting effect of formalin upon the colour-reaction given by quinone with certain proteins and other amino- and imino- compounds, and its inability to precipitate the compounds of quinone with α-proteoses, although it readily precipitates the proteoses themselves, confirm the view that it is the $-NH_2$ and $=NH$ groups present in the proteins and their hydrolytic products that react with the quinone. It is difficult to understand, however, why the products of formalisation of gelatin, ammonia, and aniline should behave exceptionally in yielding a coloration with quinone.

In order to attempt to understand the mechanism of the interaction of quinone and proteins it is necessary here to set forth the course of the reaction known to occur between an amine, such as aniline, and the above ketone.

The products of the reaction depend upon the experimental conditions, as indicated below.

1. As a result of the action of aniline upon an alcoholic solution of quinone there are three products:

1. 2. 3.



Quinone. Di-anilido-quinone. Di-anilido-quinone-anil. Di-anilido-quinone-di-anil.

2. In the presence of acetic acid the chief product is di-anilido-quinone-anil.

3. By fusing quinone with aniline and its hydrochloride the chief product is di-anilido-quinone-di-anil.

There are thus two possible reactions between quinone and proteins and their hydrolytic products:

1. The condensation of the ketonic oxygen atoms with the hydrogen of the amino- and imino-groups, forming compounds of the anil-type (see above 2, 3).

2. The replacement of hydrogen attached to the benzene nucleus of quinone by amino-acid residues through the amino- or imino-groups forming compounds of the anilido-type (see above 1, 2, 3).

These two reactions might proceed simultaneously forming compounds analogous to di-anilido-quinone-di-anil.

(iv) *Experiments with quinone-dioxime.*

Attempts were next made to discover to which type of chemical reaction the red colorations given by quinone with proteins were due.

It was thought that experiments with quinone-dioxime could decide this question, because, although substitution in the ketonic groups naturally prevents their condensation with amino- and imino-compounds, it is known not to inhibit the entrance of these substances through their nitrogen atoms into other parts of the quinone nucleus.

Quinone-dioxime was prepared by the action of hydroxylamine hydrochloride upon quinone, the reaction being carried out in acid solution to prevent the reduction of the quinone to quinol.

It was found that aqueous solutions of quinone-dioxime gave no colorations with serum, gelatin, proteoses and alanine. The gelatin after immersion in the quinone-dioxime solution was found to be still soluble in hot water and precipitated by phenol as a white substance, and was thus not chemically altered.

These results strongly suggest that the colour-reaction given by proteins with quinone is due to the condensation of the $-NH_2$ or $=NH$ groups with the ketonic groups of the quinone, compounds similar to quinone-dianil being produced. This conclusion is supported by the fact that no oxime could be obtained by the treatment of the quinone-proteose compounds with hydroxylamine hydrochloride.

The chemical action of quinone upon proteins thus resembles that of formaldehyde.

(v) *The relations of acetone to proteins.*

Since it was found that quinone possessed a germicidal power more than 100 times as great as that of acetone [Cooper, 1912, 1] it was of interest to compare the effects of these two ketones on proteins.

Acetone was found to differ from quinone in exerting a precipitating action upon proteins, but while 0·1 per cent. solutions of quinone gave a colour-reaction with egg-albumin and gelatin, the albumin was not precipitated by aqueous solutions of acetone below 12 per cent. and gelatin was not even affected by immersion in 50 per cent. and 90 per cent. solutions.

The gelatin was still soluble in hot water and precipitable by phenol after this treatment, so that there was no evidence that it was chemically altered by the acetone. This ketone is therefore to be classed with the alcohols and phenols as a protein-precipitant.

III. *The relation of the chemical reactivity of quinone towards proteins to its germicidal power.*

The fact that as a germicide quinone is greatly superior to many other para-di-substitution products of benzene is seen from the following table [Cooper, 1912, 1 and Morgan and Cooper, 1912].

Substance	Organism	Carbolic coefficient
Quinone	*Staphylococcus py. aur.*	10
Benzaldehyde ...	*B. typhosus*	10
Quinol	,,	1
p Cresol	*Staphylococcus py. aur.*	2·4
,, ...	*B. typhosus*	2·6
Aniline	*Staphylococcus py. aur.*	0·5
,, ...	*B. typhosus*	0·57
p-Toluidine ...	,,	1·25
p-Phenylene-diamine	,,	Under 0·3
p-Nitrophenol ...	*Staphylococcus py. aur.*	2·3

This itself suggests that the high germicidal power of quinone is associated with its chemical reactivity.

This conclusion is supported by certain other facts.

1. Not only is quinone superior to phenol, *p*-cresol, quinol, *p*-nitrophenol and acetone in germicidal power, but it can exert a chemical action upon proteins in concentrations (e.g. 0·1 per cent.) much lower than those in which the above substances can induce protein precipitation [Cooper, 1912, 2; 1913].

2. Benzaldehyde, which resembles quinone in its chemical action upon proteins, is also approximately equal to quinone in germicidal power, its carbolic coefficient with *B. typhosus* being 10.

There is thus some evidence that the mechanism of the germicidal action of quinone consists in a chemical interaction with some constituent protein or proteins of the bacteria essential for vitality, and not, as seems to be the case with the phenols, in a precipitating effect upon the colloidal suspension. The superiority of quinone as a germicide to various phenols and to acetone is sufficiently explained by the fact that it reacts with proteins in concentrations much lower than those in which the phenols and acetone exert a precipitating action.

SUMMARY.

1. The observations of Würster and Raciborski that quinone solutions gave a red coloration with various proteins and amino-acids have been confirmed.

2. The proteins (egg-albumin, proteins of horse serum, gelatin, Witte's peptone) could be isolated in the coloured condition from the red solutions by means of various precipitants and could not be decolorised by prolonged washing with water or alcohol. Other physical properties of the proteins, e.g. solubility, precipitability, were frequently changed as a result of the treatment with quinone, as is also the case when proteins react with formaldehyde. From these results it appeared that the proteins had become chemically altered by the quinone.

3. The colour-reaction did not occur when gelatin and proteoses were immersed in solutions of quinone in absolute alcohol. It would appear that the quinone was dissolved by the colloids before the chemical reaction, so that an efficient solvent for this ketone such as alcohol, by decreasing the uptake, could inhibit the colour-reaction.

4. The addition of sufficient formaldehyde to proteins, proteoses, amino-acids, and imino-compounds either before or simultaneously with the addition of the quinone completely inhibited the colour-reactions. Smaller amounts of formalin decreased the intensity of the red colorations. Gelatin, aniline, and ammonia however behaved exceptionally inasmuch as they still gave the colorations with the quinone after the addition of formalin. The positive results appeared not to be due to incomplete formalisation, since the isolated compounds of aniline and ammonia with formalin gave colour-reactions with quinone.

The inhibitory effect of formalin upon the colour-reactions given by quinone with certain proteins, with proteoses and amino-acids indicates that the latter react with quinone through their $-NH_2$ or $=NH$ groups.

5. Proteins, proteoses, and alanine gave no colour-reaction with quinone-dioxime, and no oxime could be prepared from the quinone-proteose compounds. This is presumptive evidence that the constituent $-NH_2$ or $=NH$ groups of the proteins and their hydrolytic products condense with the ketonic groups of the quinone. The chemical action of the latter upon proteins thus resembles that of formaldehyde.

6. Acetone differed from quinone in acting as a protein-precipitant.

7. There is some evidence that the germicidal power of quinone is due

to its chemical action upon some constituent protein or proteins of the bacterium essential for vitality and that the superiority of quinone as a germicide to phenol, quinol, and acetone is explained by its reactivity towards proteins in much lower concentration.

I desire to express my best thanks to Prof. Martin, F.R.S., for helpful criticisms of this work.

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ON THE PROTECTIVE AND CURATIVE PROPERTIES OF CERTAIN FOODSTUFFS AGAINST POLYNEURITIS INDUCED IN BIRDS BY A DIET OF POLISHED RICE.

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The historical aspect of beri-beri has recently been so exhaustively dealt with by Schaumann (1910), Simpson and Edie (1911), and Funk (1912), that it is only necessary to consider here in detail the previous investigations of the protective and curative effects of various foodstuffs and their extracts towards this disease and the analogous polyneuritis of birds.

The evidence obtained by Eijkman (1897) and Braddon conclusively proved that beri-beri occurred amongst those races that used a highly polished (steam-milled) rice as their staple diet, but not amongst those peoples whose diet consisted of rice decorticated by hand or parboiled rice.

Fletcher (1907) by the application of this discovery found that beri-beri disappeared from amongst the inmates of an asylum subsequently to the replacement of polished rice by whole rice as the staple diet.

Eijkman (1897) first shewed that a disease (polyneuritis) similar in many respects to human beri-beri could be induced in birds by polished rice diets. He furthermore found that fowls developed symptoms of this disease when fed almost exclusively on starch (tapioca, sago, etc.) and that the birds recovered after receiving raw meat.

Eijkman (1906) also found that an aqueous extract of rice-polishings cured polyneuritis after the removal of the phytin and that the curative constituent was dialysable and was not precipitated from aqueous solution by alcohol. More recently (1910) he has shewn that the curative substance of yeast is also extracted by strong alcohol.

Gryns (1900, 1909) confirmed many of Eijkman's observations and also shewed that the addition of katjang-idjoe beans and meat to the polished rice diet prevented polyneuritis. He found, however, that their protective action was destroyed by heating to 120° C.

Schaumann (1910) shewed that the addition of proteins, glycerophosphates and inorganic salts to the polished rice diets did not prevent polyneuritis in pigeons and that the addition of sodium nucleate only delayed the occurrence of the symptoms and decreased their severity. He compared quantitatively the preventive powers of dried brewers' yeast, rice-bran and wheat-bran against pigeon polyneuritis and found that the daily addition of 1·5, 2 and 7·5 gms. respectively of these substances to the polished rice diet protected the birds from this disease and led to an increase in their weights.

Schaumann also shewed that impure yeast-nucleic acid and egg-lecithin possessed slight curative properties towards polyneuritis, but could not bring about the complete recovery of the birds, while dried yeast, peas, and katjang-idjoe beans were able to restore completely birds affected with this disease. The birds could also be cured by hydrochloric acid (0·3 %) and alcoholic (96 %) extracts of these beans.

Fraser and Stanton (1910, 1911) extracted the constituent of rice-bran, which cured polyneuritis in pigeons, by dilute hydrochloric acid and absolute alcohol, and Chamberlain and Vedder (1911) further shewed that the curative substance could be removed from the dried alcoholic extract of rice-bran by water and that it was dialysable, thus confirming Eijkman's observations of years before.

Chamberlain and Vedder also found that fowls, receiving daily in addition to the polished rice diet sufficient of this aqueous extract, did not develop polyneuritis and that the extract contained only traces of phosphorus ($\frac{1}{1000}$th part of the phosphorus-content of rice-polishings). This result lent no support to the theory that beri-beri is due to a deficiency of phosphorus in the rice diet which had been advanced in more than one quarter.

Cooper and Funk (1911) confirmed many of the previous observations and found that polyneuritis could be induced in birds by diets of various pure carbohydrates, thus excluding intoxication as a cause of beri-beri. They also found that the anti-neuritic constituent of rice-polishings after extraction with acidulated alcohol and water was completely precipitated by phospho-tungstic acid and was a nitrogenous body which contained no phosphorus.

Funk (1911) has continued these fractionations and has found that after precipitation with phospho-tungstic acid and decomposition of the precipitate with baryta the curative constituent of polishings is completely precipitated by silver nitrate, partially by mercuric chloride in alcoholic solution and not at all by platinum chloride in alcoholic solution and is a crystalline body melting at 233° C. Funk has provisionally ascribed the formula $C_{17}H_{19}O_7N_2$ to the active substance.

Although Chamberlain and Vedder (1911) found that lime-juice itself did not cure polyneuritis, Funk in a later communication (1912) shewed that by concentration and fractionations similar to those described previously by him (1911) a fraction could be obtained from the lime-juice with silver nitrate possessing marked curative properties. Curative fractions were also obtained from ox-brain, yeast and milk.

Schaumann (1912) has confirmed some of the main observations of Funk and suggests that the anti-neuritic substance is an activator by which the assimilation of phosphorus is rendered possible, so that under dietetic conditions leading to the depletion in the organism of the active substance phosphorus starvation takes place.

B. Moore and his collaborators (1912) have also confirmed some of Funk's observations and have employed similar methods for the isolation from yeast of the anti-neuritic substance to which they ascribe provisionally the formula $C_7H_{17}N_2O_5$.

The investigation about to be described may be divided into two parts.

Part I deals with the quantitative determination of the preventive powers of various common foodstuffs against polyneuritis induced in birds by diets of polished rice.

Part II deals with the fractionation of some of these foodstuffs carried out with a view to the isolation of the active constituent or constituents.

Part I of the investigation was undertaken not merely on account of its intrinsic interest to dietetics, but also because of its practical importance in dealing with beri-beri. The active substance has, I believe, already been isolated by Funk at this Institute, but it exists in such minute amount and is so difficult to separate that its extraction in the pure condition for addition to a diet otherwise inadequate is not yet a practical proposition. It is likely that when its precise constitution has been determined, it may be discovered how to synthesise it but this will probably occupy some considerable time.

In the meanwhile knowledge of the relative available concentration of the active substance in certain common foodstuffs which can be used to supplement the diets of coolies will surely be of value.

Part I.

The distribution of the substance or substances preventing polyneuritis amongst foodstuffs.

The amounts of various foodstuffs have been determined which are necessary to prevent the occurrence of polyneuritis for a definite time in adult pigeons of an average weight of 350 gms. fed on polished rice[1]. As pigeons fall ill with polyneuritis in about three weeks when fed exclusively on polished rice, the standard time selected was 50 days.

Series of pigeons received in addition to the rice diet a certain ration of a foodstuff daily. The latter was administered by a crop-tube and differed in amount for each series. In this way it was possible to determine a maximum daily amount of a foodstuff insufficient to prevent polyneuritis in pigeons for 50 days and a minimum amount sufficient for this purpose. The symptoms of polyneuritis, induced by an exclusive diet of polished rice or by mixed diets consisting of polished rice and insufficient rations of the various foodstuffs, were accompanied by fatty degeneration in the sciatic nerves which was demonstrated by means of Marchi's method. No relation was observed between the degree of degeneration and the intensity of the paralytic symptoms.

The birds were weighed weekly in order to compare the influence of the various mixed diets upon their body-weights.

The anti-neuritic values of the following raw foodstuffs have been examined in this way:

1. Meat (lean beef).
2. Ox-heart.
3. Sheep-brain.
4. Fish (hake).
5. Egg-yolk.
6. Brewers' yeast.
7. Lentils.
8. Barley.

Control experiments with polished rice.

Nine pigeons (Series I) were fed exclusively on polished rice the daily ration being $\frac{1}{20}$th their body-weight. For the first two weeks of the experiments the birds ate the rice readily, but subsequently they became tired of the diet and ate very small amounts of rice, the daily ration being much less than $\frac{1}{20}$th.

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[1] Daily ration of rice = $\frac{1}{20}$th body-weight approx. During the first two weeks of an experiment the birds ate the rice naturally, but subsequently they grew tired of the diet and were fed artificially.

A second series of pigeons received by regular artificial feeding *throughout* the experiment daily rations of polished rice of from $\frac{1}{11}$th to $\frac{1}{30}$th the initial body-weight.

The results of these experiments are set forth below :

TABLE I.

Series I.

Natural feeding.

	Pigeon 1	Pigeon 2	Pigeon 3	Pigeon 4	Pigeon 5
Effect of diet upon pigeons' health	Weakness appeared on 18th day. Acute symptoms of polyneuritis on 24th day.	Symptoms of polyneuritis on 21st day.	Symptoms of polyneuritis on 22nd day.	Symptoms of polyneuritis on 32nd day.	Symptoms of polyneuritis on 33rd day.
Mean change in weight by end of a certain time	− 24·6 % after 18 days				

	Pigeon 35	Pigeon 38	Pigeon 39	Pigeon 41
Effect of diet upon pigeons' health	Symptoms of polyneuritis on 21st day.	Symptoms of polyneuritis on 28th day.	Symptoms of polyneuritis on 26th day.	Symptoms of polyneuritis on 33rd day.
Change in wt. by end of exp.	− 18·7 %	− 36 %	− 30 %	− 10·7 %

Series II.

Artificial feeding.

(*a*)

Daily ration = $\frac{1}{15}$th body-weight.

	Pigeon 181	Pigeon 182	Pigeon 184
Effect of diet upon pigeons' health	Symptoms of polyneuritis on 14th day.	Symptoms of polyneuritis on 13th day.	Symptoms of polyneuritis on 10th day.
Change in weight by end of exp.	+ 3 %	− 7·7 %	− 6 %

(*b*)

		Pigeon 241	Pigeon 240	Pigeon 239	Pigeon 236
Daily ration of rice in fractions of	Initial body-weight	1/30th	1/23rd	1/19th	1/17th
	Final body-weight	1/24th	1/15th	1/14th	1/13th
Day of exp. upon which symptoms of polyneuritis appeared		16th day	19th day	15th day	16th day
% change in weight by end of exp.		− 21·1 %	− 35·2 %	− 23·7 %	− 23 %

		Pigeon 233	Pigeon 238	Pigeon 243	Pigeon 244
Daily ration of rice in fractions of	Initial body-weight	1/14th	1/14th	1/11th	1/11th
	Final body-weight	1/13th	1/12th	1/9th	1/10th
Day of exp. upon which symptoms of polyneuritis appeared		16th day	13th day	26th day	9th day
% change in weight by end of exp.		− 7 %	− 14·3 %	− 22·4 %	− 13 %

The results indicate that the pigeons fed naturally and therefore on small rations of polished rice developed symptoms of polyneuritis from three to four weeks after being placed on the rice diet and by that time had suffered a loss in weight which varied from 10 to 36 %.

In the case of the birds receiving the larger rations by regular artificial feeding the symptoms of polyneuritis appeared much earlier. No appreciable difference, however, was noticed in the periods elapsing before the appearance of polyneuritis in the case of pigeons receiving artificially daily rations of rice varying from $\frac{1}{11}$th to $\frac{1}{30}$th the initial body-weight. The explanation of this may be that the birds eating small insufficient rations of polished rice depend upon their own tissues for making up the deficiency in the food supply. In this way they are more able than the well-fed birds to make use of the anti-neuritic substance distributed throughout their bodies and thus remain free from polyneuritis for a longer period.

Similar experiments carried out by other workers have, however, yielded most divergent results: Maurer (1907) found that fowls which ate polished rice in the largest amount were the first to fall ill with polyneuritis, and that this disease was postponed for a long time in under-fed birds. The experiments described above confirm Maurer's results. Chamberlain, Bloombergh and Kilbourne (1911), on the other hand, found that birds, which ate their full daily ration of rice, remained healthy for a longer period than those which ate less freely.

The explanation of these discordant results is not apparent.

Since wide variations in the amount of rice received daily by pigeons did not lead to any regular differences in their susceptibilities to polyneuritis, it was not necessary to ensure by artificial feeding the constancy of the daily rice ration in the quantitative determination of the anti-neuritic values of foodstuffs.

(i) *Meat* (*lean beef*). (Water content = 75 %.)

Five series of pigeons, *A*, *B*, *C*, *D*, and *E*, each series consisting of five birds, received daily in addition to the rice diet, 2, 4, 6, 10, and 20 gms. of beef respectively.

The results of these experiments are set forth in the following table:

TABLE II. *Meat* (*raw beef*).

A. Polished rice + 2 gms. beef daily.

	Pigeon 6	Pigeon 7	Pigeon 8	Pigeon 9	Pigeon 10
Effect of diet upon pigeons' health	Weakness in limbs appeared on 22nd day. Acute symptoms of polyneuritis on 27th day.	Acute symptoms of polyneuritis developed on 27th day.	Weakness in limbs appeared on 27th day. Died on 30th day with no acute symptoms of polyneuritis.	Weakness in limbs appeared on 27th day. Died on the 31st day with only slight symptoms of polyneuritis.	Weakness in limbs appeared on 27th day. Symptoms became no more acute. Death on 32nd day.
	Fatty degeneration indicated in the sciatic nerves to various extents.				
Mean change in wt. by end of a certain time	−20·9 % after 27 days				

B. Polished rice + 4 gms. beef daily.

	Pigeon 11	Pigeon 12	Pigeon 13	Pigeon 14	Pigeon 15
Effect of diet upon pigeons' health	Weakness in limbs on 22nd day. Died. Acute symptoms of polyneuritis developed on 25th day.	Weakness in limbs on 25th day. Died on 26th day without acute symptoms of polyneuritis.	Weakness in limbs appeared on 25th day. Died on 26th day without acute symptoms of polyneuritis.	Acute symptoms of polyneuritis developed on 27th day.	Weakness in limbs appeared on 33rd day. Died on next day without acute symptoms of polyneuritis.
	Fatty degeneration indicated in the sciatic nerves to various extents.				
Mean change in wt. by end of a certain time	−20·7 % after 20 days				

C. Polished rice + 6 gms. beef daily.

	Pigeon 16	Pigeon 17	Pigeon 18	Pigeon 19	Pigeon 20
Effect of diet upon pigeons' health	Acute symptoms of polyneuritis developed on 27th day.	Weakness in limbs on 27th day. Acute symptoms of polyneuritis developed on 28th day.	Severe weakness in limbs appeared on 32nd day. Died on the same day without acute symptoms of polyneuritis.	Died without symptoms on 33rd day.	Severe weakness appeared in limbs on 34th day. Died on same day without acute symptoms of polyneuritis.
	Fatty degeneration indicated in the sciatic nerves to various extents.				
Mean change in wt. at the end of a certain time	−16 % after 27 days				

D. Polished rice + 10 gms. beef daily.

<table>
<tr><th></th><th>Pigeon 21</th><th>Pigeon 22</th><th>Pigeon 23</th><th>Pigeon 24</th><th>Pigeon 25</th></tr>
<tr><td rowspan="3">Effect of diet upon pigeons' health</td><td colspan="5">Flight impaired on about the 25th day.</td></tr>
<tr><td>Weakness in limbs on 32nd day. Died on same day without acute symptoms of polyneuritis.</td><td>Weakness in limbs appeared on 32nd day.</td><td>Weakness in limbs on 38th day. Died on same day without acute symptoms of polyneuritis.</td><td>Weakness in limbs appeared on 32nd day. Died on 34th day without acute symptoms of polyneuritis.</td><td>Weakness in limbs and slight symptoms of polyneuritis developed on 59th day. Died on next day.</td></tr>
<tr><td colspan="5">Fatty degeneration indicated in the sciatic nerves to various extents.</td></tr>
<tr><td>Mean change in wt. at the end of a certain time</td><td colspan="5">−7·4 %
after 30 days</td></tr>
</table>

E. Polished rice + 20 gms. beef daily.

	Pigeon 42	Pigeon 43	Pigeon 44	Pigeon 45
Effect of diet upon pigeons' health	Remained quite healthy for 6 weeks. At end of this time ration of meat reduced to 15 gms. After another 28 days typical symptoms of polyneuritis set in.	Died on the 29th day without symptoms.	Remained quite healthy for 6 weeks. At end of this time meat ration reduced to 15 gms. After another 52 days had not developed symptoms of polyneuritis.	Remained quite healthy for 6 weeks. At end of this time meat ration reduced to 15 gms. After another 52 days had not developed symptoms of polyneuritis.
Change in wt. by end of exp.	−3·9 % (after 1st 6 weeks). −16·9 % (at end of exp.).	0	+15·6 % (after 1st 6 weeks). +12·5 % (at end of exp.).	−7·3 % (after 1st 6 weeks). −8·8 % (at end of exp.).

These results indicate that it was necessary to add 20 gms. of raw lean beef daily to the polished rice diet to prevent the occurrence of symptoms of polyneuritis in pigeons for 50 days. This amount of meat daily was also sufficient to prevent almost entirely the loss in weight which results from an exclusive polished rice diet and in the case of one pigeon it enabled a considerable increase in weight to take place.

The addition of smaller amounts of meat to the rice diet did not prevent the loss in weight and was only sufficient either to delay the occurrence of the symptoms of polyneuritis for a short time or to decrease their severity.

The results of these experiments are of practical importance insomuch as they indicate the small value of meat in the prevention and treatment of beri-beri.

(ii) *Ox-heart.* (Water-content = 60 %.)

Four series of pigeons, *A*, *B*, *C*, and *D*, each series consisting of two birds, received daily in addition to polished rice 5, 10, 15 and 20 gms. of heart-muscle (free from fat) respectively.

The results of these experiments are tabulated below:

TABLE III.

	A. Rice + 5 gms. heart.		B. Rice + 10 gms. heart.	
	No. 189	No. 190	No. 191	No. 192
Effect of diet upon pigeons' health	Quite healthy after 50 days. On 51st day daily ration of heart reduced to 2 gms. Polyneuritis appeared 20 days afterwards.	Quite healthy after 50 days. On 51st day daily ration of heart reduced to 2 gms. Polyneuritis appeared 18 days afterwards.	Quite healthy after 50 days.	Quite healthy after 50 days.
Change in wt. by end of exp.	After 50 days −3 %. After 70 days −10 %.	After 50 days −6·5 %. After 68 days −16 %.	+10·5 %	+5·7 %

	C. Rice + 15 gms. heart.		D. Rice + 20 gms. heart.	
	No. 193	No. 194	No. 195	No. 196
Effect of diet upon pigeons' health	Quite healthy after 50 days.	Quite healthy after 50 days.	Quite healthy after 50 days.	Quite healthy after 50 days.
Change in wt. by end of exp.	−3 %	−1·4 %	−6·6 %	−3 %

The results indicated that the addition of 5 gms. of heart daily to the polished rice diet was quite sufficient to prevent symptoms of polyneuritis for 50 days and also to prevent any appreciable loss in weight.

Since the daily addition of as much as 20 gms. of beef to the rice diet was necessary to prevent polyneuritis for the same period, heart-muscle was considerably superior to voluntary muscle in anti-neuritic power.

The richness of the mammalian heart in the substance preventing polyneuritis may be correlated with the fact that in human beri-beri heart failure accompanied by fatty degeneration is a very common symptom.

(iii) *Sheep-brain.* (Water-content = 80 %.)

Three series of pigeons, A, B, and C, each series consisting of three birds, received daily in addition to the polished rice diet 3, 6, and 15 gms. of sheep-brain respectively.

The results of the experiments are set forth in the following table:

TABLE IV. *Polished rice + Sheep-brains (raw).*

A. Polished rice + 3 gms. brains daily.

	Pigeon 97	Pigeon 98	Pigeon 99
Effect of diet upon pigeons' health	Symptoms of polyneuritis on 21st day.	Symptoms of polyneuritis on 24th day.	Died on 66th day with symptoms of weakness.
Change in weight by end of exp.	– 12 %	– 10 %	0 %

B. Polished rice + 6 gms. brains daily.

	Pigeon 100	Pigeon 101	Pigeon 102
Effect of diet upon pigeons' health	Died on 45th day without symptoms.	Symptoms of polyneuritis on 20th day.	Died on 66th day with symptoms of weakness.
Change in weight by end of exp.	– 3 %	– 3 %	– 12·5 %

C. Polished rice + 15 gms. brains daily.

	Pigeon 103	Pigeon 104	Pigeon 105
Effect of diet upon pigeons' health	Still healthy after 43 days. Daily ration of brain then reduced to 8 gms. Symptoms of polyneuritis 47 days later.	Still healthy after 43 days. Daily ration of brain then reduced to 8 gms. Still healthy after another 47 days.	Still healthy after 43 days. Daily ration of brain then reduced to 8 gms. Still healthy after another 47 days.
Change in weight by end of exp.	End of 1st period (43rd day) – 12·6 %. End of exp. (90th day) – 26·6 %.	End of 1st period (43rd day) + 9·4 %. End of exp. (90th day) – 4·7 %.	End of 1st period (43rd day) – 4 %. End of exp. (90th day) – 21 %.

The experiments indicated that in some cases the daily addition of 3 and 6 gms. of brain to a diet of polished rice delayed the appearance of symptoms of polyneuritis, but to prevent consistently their occurrence for 50 days a daily ration of as much as 12 gms. of brain (the mean of 8 and 15 gms.) was necessary.

Considering the great importance of the anti-neuritic substance in the activities of the nervous system its low concentration in brain is remarkable. This may indicate that in metabolism it is constantly being converted into substances, which do not possess anti-neuritic properties, so that its concentration in the nervous system must always remain small.

The effect of brain-material in maintaining body-weight was very much greater, being demonstrable even when the anti-neuritic substance was present in insufficient amount.

It was previously found that in the case of birds fed exclusively on polished rice considerable loss in weight could only be prevented by giving extra large daily rations.

These results indicate a secondary deficiency in polished rice, namely, that of substances necessary for the maintenance of the body-weight and evidently contained in comparatively large amount in brain. Considerable fatty degeneration was indicated by Marchi's method in the sciatic nerves of the birds which developed polyneuritis without fall in body-weight. The degeneration was usually quite as extensive as that occurring in pigeons in which great loss in weight accompanied the polyneuritis. It would appear that the fatty degeneration is an effect of the deficiency in polished rice of the anti-neuritic substance and not of the secondary deficiency of substances essential for the maintenance of body-weight.

(iv) *Fish*. (Water-content = 80 %.)

Raw hake-muscle was used in the experiments.

Two series of pigeons, each series consisting of four birds, received respectively in addition to the polished rice diet 5 and 10 gms. of fish daily.

The results of these experiments are set forth below:

TABLE V. *Fish* (*Hake*).

Polished rice + 5 gms. fish daily.

	Pigeon 46	Pigeon 47	Pigeon 48	Pigeon 49
Effect of diet upon pigeons' health	Died on 54th day of exp. without symptoms of weakness or polyneuritis.	Developed symptoms of polyneuritis on 20th day.	Developed symptoms of polyneuritis on 30th day.	Developed symptoms of general weakness on 21st day which were followed by acute symptoms of polyneuritis the next day.
Change in wt. by end of exp.	– 22·5 %	– 26·4 %	– 23 %	– 38·3 %

Polished rice + 10 gms. fish daily.

	Pigeon 50	Pigeon 51	Pigeon 52	Pigeon 53
Effect of diet upon pigeons' health	Developed symptoms of general weakness on 21st day which passed to those of polyneuritis on 23rd day.	Developed symptoms of polyneuritis on 23rd day.	Developed symptoms of polyneuritis on 24th day.	Developed symptoms of polyneuritis on 31st day.
Change in wt. by end of exp.	– 23·9 %	– 26·6 %	– 24·6 %	– 28 %

The results indicated that the addition of as much as 10 gms. of fish daily to the polished rice diet neither delayed the occurrence of polyneuritis nor arrested the fall in weight that results from exclusive rice diets. Fish muscle therefore resembled meat in being deficient in the anti-neuritic substance.

(v) *Egg-yolk.* (Water-content = 50 %.)

Four series of pigeons, *A*, *B*, *C*, and *D*, each series consisting of two birds, received daily in addition to polished rice 1, 2, 5 and 10 gms. respectively of raw egg-yolk.

The effect of cooking upon the anti-neuritic power of egg-yolk was also investigated. The eggs were boiled for 4 minutes and the coagulated yolks were ground with water and administered to the birds by means of a crop-tube.

Two series of pigeons, *E* and *F*, each series consisting of two birds, received daily 3 and 6 gms. respectively of cooked yolk in addition to the rice diet.

The results of the experiments are tabulated below:

TABLE VI. *Polished rice + Raw egg-yolk.*

	A. Pol. rice + 1 gm. yolk daily.		B. Pol. rice + 2 gms. yolk daily.	
	Pigeon 185	Pigeon 186	Pigeon 187	Pigeon 188
Effect of diet upon pigeons' health	Symptoms of polyneuritis on 26th day.	Symptoms of polyneuritis on 12th day.	Symptoms of weakness in limbs on 43rd day. On increasing the daily ration of yolk to 4 gms. the symptoms were prevented from becoming more acute.	Symptoms of weakness in limbs on 41st day. Ration of egg increased to 3 gms. Symptoms had entirely disappeared before 50th day and loss in weight was arrested.
Change in wt. by end of exp.	− 21·9 %	− 17·6 %	By 43rd day − 16·7 %. By 68th day − 28·2 % (end of exp.).	By 41st day − 24·3 %. By 50th day − 21·4 % (end of exp.).

	C. Pol. rice + 5 gms. yolk daily.		D. Pol. rice + 10 gms. yolk daily.	
	Pigeon 136	Pigeon 137	Pigeon 138	Pigeon 139
Effect of diet upon pigeons' health	Still healthy after 55 days.	Still healthy after 55 days.	Still healthy after 55 days.	Still healthy after 55 days.
Change in wt. by end of exp.	− 30·7 %	− 32·1 %	− 7·5 %	− 3 %

	E. Pol. rice + 3 gms. cooked yolk daily.		F. Pol. rice + 6 gms. cooked yolk daily.	
	Pigeon 201	Pigeon 202	Pigeon 203	Pigeon 204
Effect of diet upon pigeons' health	Still healthy after 50 days.	Still healthy after 50 days.	Still healthy after 50 days.	Still healthy after 50 days.
Change in wt. by end of exp.	– 25·7 %	– 24·4 %	– 12·2 %	– 19 %

The daily addition of 5 gms. of raw egg-yolk or of 3 gms. of the cooked yolk to the polished rice diet therefore prevented the occurrence of symptoms of polyneuritis for 50 days and 2 gms. of raw yolk daily delayed the appearance of the symptoms until about the 40th day of the experiment. These disappeared when the ration of egg-yolk was increased to 3 or 4 gms.

The results indicate that the addition of 3 gms. of raw egg-yolk daily to the polished rice would be just sufficient to prevent the occurrence of symptoms of polyneuritis for 50 days.

It is seen that the daily ration of boiled egg-yolk (3 gms.) sufficient for this purpose was about midway between the insufficient daily ration of raw egg-yolk (2 gms.) and the 5 gms. allowance that prevented the occurrence of polyneuritis symptoms for 50 days. It may be concluded from these results that the process of heating at 100° C. for 4 minutes had no measurable effect upon the anti-neuritic value of egg-yolk.

A comparison of the amounts of beef, ox-heart, brain, and egg-yolk, which, when added daily to the rice diet, were sufficient to prevent symptoms of polyneuritis for 50 days, indicates that in the natural condition egg-yolk considerably exceeds the other foodstuffs in its capacity for preventing this disease.

(vi) *Dried brewers' yeast.* (Water-content = 5 %.)

Pressed yeast was sieved and then dried at 37° C. for 2 days. After this treatment it only contained 5 % of moisture. Three series of pigeons, *A*, *B*, and *C*, each series consisting of two or three birds, received daily $2\frac{1}{2}$, 1, and $\frac{1}{2}$ gms. respectively of this dried yeast preparation in addition to polished rice. The results of these experiments are set forth below.

These experiments indicate that dried brewers' yeast possessed considerable preventive power against polyneuritis. One-half a gram of this preparation added to the polished rice diet was more than sufficient to maintain pigeons free from this disease for 50 days and also to prevent any loss in weight.

As Schaumann (1911) found that the addition of an alcoholic extract of yeast to the polished rice diet, although it prevented polyneuritis, did not prevent the loss in weight, the constituents of yeast that can maintain the body-weight are evidently insoluble in alcohol.

TABLE VII. *Polished rice + Yeast (dried).*

	A. Pol. rice + 2½ gms. dried yeast.		B. Pol. rice + 1 gm. dried yeast.	
	Pigeon 73	Pigeon 74	Pigeon 69	Pigeon 83
Effect of diet upon pigeons' health	At end of 40 days was still healthy.	At end of 40 days was still healthy.	At end of 59 days was still healthy. Daily allowance of yeast then reduced to ½ gm. and after another 52 days bird was still healthy but had lost in weight.	At end of 54 days was still healthy. After 54 days yeast was stopped and bird received only rice. It developed symptoms of polyneuritis 27 days subsequently.
Change in wt. by end of exp.	+13 %	+1·4 %	After 1st 59 days +12·3 %. At end of exp. −4·1 %.	After 1st 54 days +8·5 %.

C. Pol. rice + ½ gm. dried yeast.

	Pigeon 68	Pigeon 81	Pigeon 82
Effect of diet upon pigeons' health	At end of 59 days was still healthy. Yeast allowance was then reduced to ¼ gm. and bird was still healthy after another 68 days although it had lost in weight.	At end of 54 days was still healthy. Yeast allowance was then stopped and bird developed symptoms of polyneuritis 14 days subsequently and had lost in weight.	At end of 54 days was still healthy. Yeast allowance was then stopped and symptoms of polyneuritis appeared 24 days subsequently and loss in weight occurred.
Change in wt. by end of exp.	After 1st 59 days +6 %. At end of exp. −12 %.	After 1st 54 days +4·7 %. At end of exp. −16·6 %.	After 1st 54 days +5·2 %. At end of exp. −23·7 %.

(vii) *Dried lentils.* (Water-content = 12 %.)

Series of pigeons, *A*, *B*, *C*, and *D*, each series consisting of 2 birds, received daily in addition to polished rice 1, 3, 4½ and 6 gms. respectively of uncooked lentils.

The results of the experiments are tabulated below.

These experiments indicate that the daily addition of 3 gms. of dried lentils to the polished rice diet was sufficient to prevent the occurrence of polyneuritis for at least 50 days, but could not prevent the loss in weight. For the maintenance of the body-weights of the pigeons 6 gms. of lentils daily were necessary.

TABLE VIII. *Polished Rice + Dried Lentils.*

	A. Pol. rice + 1 gm. lentils.		B. Pol. rice + 3 gms. lentils.	
	Pigeon 150	Pigeon 151	Pigeon 106	Pigeon 107
Effect of diet upon pigeons' health	Symptoms of polyneuritis on 38th day.	No symptoms after 39 days.	Healthy after 57 days.	Healthy after 57 days.
Change in weight by end of exp.	−37 %	−32 %	−18 %	−7 %

	C. Pol. rice + $4\frac{1}{2}$ gms. lentils.		D. Pol. rice + 6 gms. lentils.	
	Pigeon 130	Pigeon 131	Pigeon 108	Pigeon 110
Effect of diet upon pigeons' health	Healthy after 55 days.	Healthy after 55 days.	Healthy after 57 days.	Healthy after 57 days.
Change in weight by end of exp.	−14·5 %	−19 %	+26 %	+18·7 %

(viii) *Unhusked barley.* (Water-content = 12 %.)

Three series of pigeons, each series consisting of 4 birds, received daily by artificial feeding mixed diets of the following compositions:

	Series A	Series B	Series C
Polished rice	15 gms.	12 gms.	8 gms.
Barley	3 ,,	6 ,,	10 ,,
Raw beef	2 ,,	2 ,,	2 ,,

The results of the experiments are set forth in the following table:

TABLE IX.

A. Polished rice 15 gms.; Raw beef 2 gms.; Unhusked barley 3 gms. daily.

	Pigeon 54	Pigeon 55	Pigeon 56	Pigeon 57
Effect of diet upon pigeons' health	Symptoms of polyneuritis on 17th day.	Still healthy after 57 days but had lost 17·7 % in weight. Diet altered on 57th day thus: Pol. rice 13 gms., Beef 2 gms., Barley 5 gms. This led to rise in weight and after 33 days more final change in weight was −4·1 %.	Weakness in limbs on 18th day which passed to acute symptoms of polyneuritis on 26th day.	Weakness in limbs on 57th day with loss in weight of 33·3 %. On 57th day diet was altered thus: Pol. rice 13 gms., Beef 2 gms., Barley 5 gms. This led to complete disappearance of weakness and to rise in weight, the final loss after 33 days more being 25·6 %.
Change in wt. by end of exp.	−19·7 %	...	−14·3 %	...

B. Polished rice 12 gms.; Raw beef 2 gms.; Unhusked barley 6 gms. daily.

	Pigeon 58	Pigeon 59	Pigeon 60	Pigeon 61
Effect of diet upon pigeons' health	After 53 days was still healthy.	After 53 days was still healthy.	Died on the 5th day without symptoms.	After 53 days was still healthy.
Change in wt. at end of exp.	0	−13·8 °/₀	0	+30·6 °/₀

C. Polished rice 8 gms.; Raw beef 2 gms.; Unhusked barley 10 gms. daily.

	Pigeon 62	Pigeon 63	Pigeon 64	Pigeon 65
Effect of diet upon pigeons' health	After 53 days was still healthy.	Died on 11th day without symptoms.	After 53 days was still healthy.	After 53 days was still healthy.
Change in wt. at end of exp.	0	0	−9·9 °/₀	+1·3 °/₀

These experiments indicate that birds fed on a mixed diet consisting of polished rice 12 gms., meat 2 gms., barley 6 gms., did not develop symptoms of polyneuritis in 53 days and that a diet containing polished rice 13 gms., meat 2 gms., barley 5 gms., could recuperate birds in the early stages of polyneuritis resulting from diets consisting too exclusively of polished rice.

Since a daily ration of 2 gms. of meat scarcely delayed the symptoms of polyneuritis, it is probable that the addition of 5 gms. of barley to the rice diet daily is sufficient to prevent the occurrence of polyneuritis in pigeons for 50 days. Takaki (1885–87) by the addition of meat and barley to the polished rice diet was able to eradicate almost completely beri-beri from the Japanese Navy. A comparison of the anti-neuritic values of barley and meat indicates that the prevention of the disease was due to the former to a greater extent than to the latter.

The proportion of barley in the above mixed diets was also found to influence the body-weights of the birds. While the pigeons receiving only 3 gms. of barley in their daily ration all lost considerably in weight, of those receiving 6 and 10 gms. of barley daily with correspondingly smaller rations of polished rice, some lost to a small extent, some gained and others remained stationary in weight.

These results indicated that a total ration of 20 gms. of grain containing 10 to 12 °/₀ of water provided it contained a certain proportion of barley were quite sufficient to keep adult pigeons weighing about 350 gms. in nutritive equilibrium. The loss in weight that accompanies polyneuritis induced by rations of polished rice equal to the above in bulk does not appear to be due to an inadequate supply of protein or carbohydrate[1], but to a deficiency in polished rice of certain substances of at present unknown nature.

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[1] During the process of polishing rice retains its carbohydrate and nearly all the protein, but becomes deficient in fat and phosphorus.

Osborne and Mendel (1911) have shewn that, while both young and adult rats could be maintained in body-weight on diets consisting of pure proteins, carbohydrates, lard and salts, there was no indication of growth in the former. When milk or extracts of milk free from protein and fat, however, were added to the dietaries, growth was found to proceed. Hopkins (1912) has obtained similar results with young rats. It would appear that the substances essential for growth and for maintenance of body-weight are not identical.

A comparison of the preventive powers of the various foodstuffs against polyneuritis and loss in weight.

In the following table are compared the amounts of various uncooked foodstuffs required to be added daily to the polished rice diet to prevent firstly, the occurrence of symptoms of polyneuritis in pigeons of an average weight of 350 gms. for fifty days, and secondly, any appreciable loss in weight.

	Daily ration required to prevent polyneuritis		Daily ration required to prevent loss in weight	
Foodstuffs	In terms of natural foodstuff	In terms of dry-weight	In terms of natural foodstuff	In terms of dry-weight
Beef	20 gms.	5 gms.	20 gms.	5 gms.
Ox-heart ...	5	2	5	2
Sheep-brain ...	12	2·5	3 to 6	0·6 to 1·2
Fish (Hake) ...	>10	>2	>10	>2
Egg-yolk ...	3	1·5	10	5
Yeast (pressed) ...	2·5	0·5	2·5	0·5
Lentils ...	15	3	30	6
Barley Unhusked	3·75	3·25	7·5	6·5
Barley Husked...	5	4·5	10	9

The results indicate that in the natural condition yeast and egg-yolk were nearly equal in anti-neuritic value and greatly exceeded the other foodstuffs in this respect. On comparing the preventive powers in terms of dry weight, however, yeast was three times as efficient as egg-yolk.

Of the foodstuffs examined of animal origin heart-muscle and egg-yolk were the most efficient, while beef was of particularly small anti-neuritic value and, in fact, was the most inefficient of the above eight foods with the possible exception of fish. On comparing the vegetable substances yeast was found to be more potent in preventing neuritis than a dried pulse (lentils) and a husked cereal (barley), which were about equal in anti-neuritic value.

A consideration of the above table shews that the amounts of the various foodstuffs required to be added to the polished rice diet to prevent polyneuritis and to maintain the body-weight did not go hand in hand. Thus a potent anti-neuritic substance was not necessarily efficient in preventing loss in weight.

For convenience the foods can be arranged into three classes according to their relative efficiencies in preventing neuritis and in maintaining body-weight.

Class I. Foodstuffs *inefficient* in both capacities.

Polished rice, beef.

Class II. Foodstuffs *efficient* in both capacities.

Yeast, heart, brain.

Class III. Foodstuffs efficient in preventing polyneuritis but less efficient *in maintaining* body-weight.

Egg-yolk, barley, lentils.

It is concluded that the capacities of the various foodstuffs for preventing polyneuritis and maintaining body-weight are due to distinct constituents. The substances possessing the latter property are probably not proteins in general, since beef is so inefficient in preventing the loss in weight induced by the rice diet. They appear also not to be fats or lipoids, because egg-yolk which is very rich in these substances is not efficient in preventing the loss in weight. Further, polished rice is so deficient in fat (0·2 %) that its ability to prevent a great loss in weight and even to maintain the body-weight when given in *large ration* cannot be ascribed to this constituent.

As already stated Schaumann (1911) shewed that, although they are not affected with polyneuritis, birds lose weight considerably when alcoholic extract of yeast is added to the polished rice diet. With the addition of an equivalent amount of the original yeast, however, the birds are quite healthy and body-weight is maintained. He considers that the anti-neuritic substance is an activator which renders possible the assimilation of phosphorus by the organism. He further supposes that the yeast acts not only by virtue of its content of this substance, but also through its constituent phosphorus, which supplements the phosphorus-content of the rice, thus effecting a maintenance of the body-weight, and that the inability of the alcoholic extract to prevent loss in weight is due to the fact that the phosphorus-content of yeast is only partially extracted by alcohol.

As it is possible however for polyneuritis to occur even when the body-weight is maintained it would appear that the polyneuritis and loss in weight induced by diets of polished rice are due to distinct causes, the former to a deficiency in the anti-neuritic substance and the latter to a deficiency of one or more substances of at present unknown nature.

A comparison of the anti-neuritic values of the above foodstuffs indicates that egg-yolk, ox-heart, yeast, lentils and barley will be effective in the prevention of beri-beri. Lentils and barley on account of their cheapness and suitability will probably be found the most practicable for this purpose.

PART II.

The curative properties of extracts of various foodstuffs towards polyneuritis in pigeons.

The various extracts were given orally to the pigeons, exhibiting the acute symptoms of polyneuritis. At the time of receiving the doses the condition of the birds was such that untreated they would have died within 24 hours. There was often a marked improvement two or three hours after the administration of a curative extract and recovery was generally complete within 24 hours.

(*a*) *Meat* (*Beef*).

Beef juice. Raw beef was well minced and pressed by means of a screw-press. The extract was then concentrated *in vacuo* at 30° C.

In another experiment the minced beef was thoroughly mixed with sand and Kieselguhr, the mass pressed in the Buchner press and the extract concentrated *in vacuo* at 30° C.

These extracts caused no improvement in the condition of four pigeons when administered in amounts equivalent to from 3 oz. to 1½ lbs. of the original meat.

The alcoholic extract. Thoroughly minced beef was shaken for three hours with an equal weight of absolute alcohol in the shaking machine; the mixture was then pressed by means of the screw-press and the extract concentrated *in vacuo* at 30° C. Taking into consideration the high water-content of meat, the extraction was in reality carried out with 60% alcohol (approx.). Doses of the concentrated preparation equivalent to amounts of beef varying from 4·6 to 6·4 oz. were sufficient to cause the complete recovery of three birds affected with polyneuritis. Some of the concentrated alcoholic extract was extracted at ordinary temperatures with purified ether. The ethereal layer was separated and concentrated at 20° C. *in vacuo*. The residue of the alcoholic extract insoluble in ether was also evaporated *in vacuo* to remove all dissolved ether.

The ethereal extract was administered to four birds affected with polyneuritis in amounts equivalent to and even much greater than the curing dose of the original alcoholic extract and was found to possess no curative properties.

The residue of the alcoholic extract was given to a pigeon in an amount equivalent to the curing dose of the original alcoholic extract and the bird completely recovered in 24 hours except that its flight was still slightly weak.

In another experiment the meat was first of all dried at room temperature by means of the electric fan and it was then ground and extracted with absolute alcohol in the shaking machine. The filtered extract was concentrated *in vacuo* at 30° C. to remove all the alcohol. An amount of this extract equivalent to 8 oz. of meat effected the complete recovery of a bird ill with polyneuritis.

A portion of this alcoholic extract was shaken with 10 times its volume of water; the liquid was filtered and the turbid filtrate shaken with a little ether. This caused the liquid to separate into three layers, (i) A top ethereal layer, containing only a trace of solid matter which was of a fatty nature, (ii) a middle layer of a turbid character, (iii) a third clear aqueous layer.

The curing dose of the bottom aqueous layer was about equivalent to that of the original alcoholic extract.

A dose of the turbid layer equivalent to the curing dose of the original alcoholic extract caused a slight improvement in the condition of another bird, but a relapse speedily set in followed by death. The residue of the alcoholic extract insoluble in water when administered in an amount equivalent to twice the curing dose of the original alcoholic extract greatly improved the condition of two pigeons, but did not effect complete recovery, as three days later the birds were still lame and weak in flight and shortly afterwards they died. These results indicate that the curative constituent was almost entirely removed from the alcoholic extract by water. Since this substance could not be extracted by water from the original meat, it must be set free in some way during the alcoholic extraction.

The ethereal extract. The meat dried in the way already described was extracted with purified ether; the extract was filtered and the ether evaporated off at 20° C. *in vacuo.* This extract possessed curative properties against polyneuritis, but it was considerably less potent than the alcoholic extracts, since 11 gms., an amount equivalent to about one kilogram of undried meat, although it caused a marked improvement in eight hours, did not entirely remove the lameness of the bird for six days and even then the power of flight was not entirely regained.

Summary. The constituent of meat that cures polyneuritis is therefore not extracted by water and only to a small extent by ether. It is readily removed from meat by both 60 % and absolute alcohol and after the alcoholic extraction is almost completely soluble in water. This suggests that it is in some way set free during the process.

(*b*) *Egg-yolk.*

Twenty-four egg-yolks, weighing 350 gms., were extracted with 1700 c.c. of absolute alcohol in the shaking machine at ordinary temperatures. Taking into consideration the water-content of the yolk (50 %), this was equivalent to extraction with about 90 % alcohol. The extract was filtered and the alcohol removed by evaporation *in vacuo* at 30° C.

An amount of this extract equivalent to four egg-yolks completely cured a pigeon affected with polyneuritis, while a dose equivalent to seven yolks given to another pigeon greatly improved its condition but could not remove the lameness.

In a later experiment twenty egg-yolks weighing 300 gms. were dried by means of the electric fan at 20° C. and the dry ground yolk was thoroughly extracted with purified ether in the shaking machine. The ether was evaporated from the filtered extract at 20° C. *in vacuo.*

Eight gms. of the dried ethereal extract (= 3 egg-yolks) were sufficient to cure a pigeon affected with polyneuritis and to prevent the reappearance of the symptoms for 14 days. Five gms. and 7 gms. of this ethereal extract improved the conditions of pigeons in 3 to 14 hours, but could not entirely remove the lameness and flight weakness. Nevertheless these amounts were sufficient to delay the reappearance of the acute symptoms of polyneuritis for about ten days.

The residue of the egg-yolks insoluble in ether was next extracted with absolute alcohol and the filtered extract was concentrated *in vacuo* at 30° C. An amount of this extract equivalent to ten egg-yolks cured a pigeon of polyneuritis and the symptoms did not reappear until eight days afterwards.

Summary. The constituent of egg-yolk that cures polyneuritis in pigeons is therefore readily extractable with 90 % alcohol and with ether; after extraction with the latter a further yield can be obtained from the residue by means of absolute alcohol.

(*c*) *Dried lentils.*

The lentils were thoroughly ground in a coffee-mill and one kilogram was extracted at 20° C. in the shaking machine with three litres of absolute alcohol. The mixture was filtered and the residue again extracted with three litres of alcohol. The combined filtrate was concentrated *in vacuo* at 30° C. to remove all the alcohol. The dried extract, which was a yellowish-red fatty mass, weighing 30 gms. and an amount of it equivalent to 20 gms. of lentils was just sufficient to bring about the complete recovery of pigeons affected with polyneuritis.

A portion of the alcoholic extract was shaken at 40° C. with ten times its volume of water. The filtered extract was concentrated *in vacuo* at 30° C. and the concentrated liquor was found to possess curative properties.

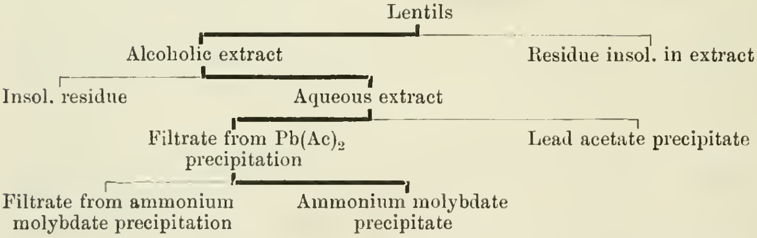
To this aqueous extract a solution of basic lead acetate was gradually added, until there was no more precipitation and the mixture was allowed to stand six hours and was then filtered. The filtrate was a dark yellow liquid with a somewhat ammoniacal odour. The traces of lead were removed from it by careful precipitation with sulphuric acid and the filtrate was concentrated *in vacuo* at 30° C. in the presence of a very slight excess of sulphuric acid. The curing dose of the concentrated filtrate was approximately equivalent to that of the original alcoholic extract. This indicates that the anti-neuritic constituent is not precipitated by basic lead acetate.

The filtrate gave no precipitate with zinc sulphate, cadmium nitrate, cobalt sulphate, uranium acetate and tannic acid, but gave a slight precipitate of a blue colour with copper sulphate, a copious white precipitate with silver nitrate and also with acetone. Ammonium molybdate yielded a voluminous yellow precipitate. To some of this filtrate a solution of ammonium molybdate was gradually added until there was no further precipitation. The mixture was allowed to stand for 12 hours and was then filtered; the precipitate was washed with water, shaken for two hours with an aqueous suspension of baryta, filtered, and the barium was removed from the filtrate with sulphuric acid. The filtrate free from baryta was concentrated *in vacuo* at 30° C. in the presence of a trace of sulphuric acid. An amount of the concentrated liquor equivalent to 100 gms. of lentils improved the condition of a bird affected with polyneuritis in three hours, but did not effect a complete recovery, as the lameness and weakness in flight had not disappeared in 48 hours and acute symptoms of polyneuritis reappeared at the end of this period. A dose equivalent to 250 gms. of lentils, however, brought about the complete recovery of another pigeon which did not develop symptoms of polyneuritis again until nine days afterwards.

The filtrate from the ammonium molybdate precipitation was carefully treated with baryta to precipitate the excess of molybdate and, after the removal of the precipitated barium molybdate by filtration, was concentrated *in vacuo* at 30° C. in the presence of a little sulphuric acid. An amount of the concentrated filtrate equivalent to 100 gms. of lentils caused no improvement in a pigeon ill with polyneuritis; an amount equivalent to 250 gms., however, possessed very slight curative properties.

These facts show that the curing constituent of lentils is almost completely precipitated by ammonium molybdate. The curative dose of the decomposed precipitate, however, was very much larger than that of the filtrate after the lead acetate fractionation. This points to the disappearance of some of the active substance during the succeeding fractionation (with molybdate). Possibly the substance was destroyed by contact with the alkali.

Summary of process of fractionation of lentils.


Lentils
Alcoholic extract
Residue insol. in extract
Insol. residue
Aqueous extract
Filtrate from Pb(Ac)$_2$ precipitation
Lead acetate precipitate
Filtrate from ammonium molybdate precipitation
Ammonium molybdate precipitate

N.B. The thick line indicates the *main* course of the curative constituents during this fractionation.

The action of strychnine upon pigeons affected with polyneuritis.

Pigeons affected with polyneuritis to such a degree that untreated they would have died within 24 hours received orally doses of strychnine varying from 0·0002 gm. to 0·025 gm. The strychnine was found to possess no curative properties, but, although the symptoms of polyneuritis remained as acute as ever, it was able to prolong the life of the birds for periods varying from two to five days. Strychnine thus appears to resemble in physiological action a crystalline body obtained by Funk (1912) from lime-juice by fractionation with silver nitrate, which was able to keep birds affected with polyneuritis alive for 72 hours without improving their condition.

It is interesting to note in this connection that daily injections of strychnine are stated to have been successful in the treatment of beri-beri.

SUMMARY.

I. *On the distribution of the anti-neuritic substance amongst foodstuffs.*

1. The amounts of various raw foodstuffs that must be added to the polished rice diet to prevent symptoms of polyneuritis in pigeons for a definite period have been determined. The results indicate that the anti-neuritic body is most irregularly distributed amongst foodstuffs.

2. Pigeons fed naturally on daily rations of polished rice of $\frac{1}{20}$th the body-weight for the first two weeks and on much smaller rations subsequently develop symptoms of polyneuritis three to four weeks after being placed on this diet and lose from 10 to 40 % in weight.

3. Pigeons fed on daily rations of polished rice of from $\frac{1}{11}$th to $\frac{1}{30}$th the body-weight develop symptoms of polyneuritis in a much shorter time (in about 14 days). The explanation of this may be that birds fed naturally on small insufficient rations have to rely on their own tissues for supplementing the food supply and are thus able to obtain for use a larger proportion of the anti-neuritic substance distributed in the tissues than the well-fed birds. Consequently the former remain free from polyneuritis for a longer period than the latter.

4. As much as 20 gms. of raw beef are necessary daily to prevent polyneuritis in pigeons weighing about 350 gms. The small anti-neuritic value of this foodstuff indicates that the addition of flesh to a diet is not likely to be very effective in preventing beri-beri.

5. The heart-muscle of the ox greatly exceeds the voluntary muscle of the same animal in its capacity for preventing polyneuritis. This is interesting as in human beri-beri heart failure is a common symptom.

6. Sheep-brain is only about twice as efficient as beef in preventing polyneuritis. The low concentration of the anti-neuritic substance in brain suggests that the substance although essential for the integrity of the nervous tissues is not contained in them as such.

7. Although the addition of small amounts of brain to a diet of polished rice does not prevent polyneuritis, it is effective in almost entirely checking the loss in weight that ensues on this diet. Since birds fed exclusively on polished rice can only be maintained in body-weight by giving very large rations, it is concluded that there is a secondary deficiency in polished rice of substances essential for the maintenance of body-weight and that these are comparatively abundant in brain.

8. Polyneuritis is accompanied by fatty degeneration in the nervous system whether body-weight is maintained or not. This indicates that the degeneration is an effect of the deficiency in polished rice of the anti-neuritic substance and not of the secondary deficiency of substances necessary for the maintenance of body-weight.

9. The daily addition of 10 gms. of fish (hake) to the rice diet did not prevent polyneuritis and loss in weight.

10. Egg-yolk exceeds all the other foodstuffs examined of animal origin in anti-neuritic value, three grams daily added to the rice diet being sufficient to prevent polyneuritis. Its capacity for preventing this disease is not measurably altered by boiling for four minutes.

11. Dried lentils and unhusked barley are efficient anti-neuritic foodstuffs. Three and five grams respectively of these substances added daily to the rice diet are sufficient to prevent polyneuritis.

12. The loss in weight induced by daily rations of polished rice equivalent to about $\frac{1}{20}$th the body-weight can be prevented without altering the actual quantity of food given by substituting barley for polished rice to the extent of about 30 %. This loss in weight is therefore shewn to be due to a genuine deficiency in polished rice and is not the effect of underfeeding.

13. One-half a gram of dried yeast daily is quite sufficient to prevent polyneuritis and also to maintain the body-weights of birds fed on polished rice.

Yeast is more efficient in preventing this disease than any of the above foodstuffs.

14. Egg-yolk, heart-muscle, yeast, lentils and barley will be effective in the prevention of beri-beri. On account of their cheapness lentils and barley will probably be the most practicable for this purpose.

15. The relative efficiencies of the various foodstuffs in preventing polyneuritis and in maintaining the body-weights of birds fed on

polished rice do not correspond. This indicates that the anti-neuritic constituent and the substances maintaining body-weight are not identical. Evidence is brought forward in the text which seems to indicate that the latter substances are not protein, fatty or lipoidal in nature.

II. *On the curative properties of extracts of various foodstuffs.*

1. *Meat.* The anti-neuritic constituent is not extracted by water and only to a small extent by ether. It is readily extracted by alcohol and after this procedure, although still insoluble in ether, is easily dissolved by water.

2. *Egg-yolk.* The curative constituent is present in a form readily extracted by both alcohol and ether. It is not however entirely removed by the latter solvent, since an alcoholic extract of egg-yolk which has been thoroughly exhausted with ether still possesses marked curative properties. These results indicate that a large portion of the anti-neuritic substances contained in meat and egg-yolk is not combined with the fats or free (ether-soluble) lipoids, but is possibly present in the *combined* lipoids, which are only extracted by alcohol.

3. *Lentils.* The constituent of lentils that cures polyneuritis is soluble in strong alcohol and in water, is not precipitated by basic lead acetate and is almost entirely precipitated by ammonium molybdate. There is evidence that the active substance disappears in large quantities during the latter fractionation. It is possibly destroyed by contact with alkali.

Strychnine. Strychnine does not cure polyneuritis but prolongs the lives of pigeons affected with this disease.

I am glad to have this opportunity of expressing my best thanks to Dr C. J. Martin, F.R.S., for much helpful advice in the course of this investigation.

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The Development of a Parasite of Earth-worms.

By JOHN WESTRAY CROPPER, M.B., M.Sc., Liverpool.

(Communicated by Sir R. Ross, K.C.B., F.R.S. Received June 20, 1912.)

(From the Laboratories of the John Howard McFadden Research Fund, Lister Institute.)

[PLATE 14.]

While carrying out experiments on the artificial induction of cell-division, I had occasion to examine the seminal vesicles of the common earth-worm, when I noticed that certain epithelial cells contained within their cytoplasm structures similar to those known as "Kurloff's bodies" found in the blood-cells of guinea-pigs.

These Kurloff's bodies have recently been shown to be parasites, to which the name of *Lymphocytozoon cobayæ* has been given. In their early stages, they are parasites of the mononuclear cells (lymphocytes) of the blood of the guinea-pig. As described in the paper on the subject (E. H. Ross, 1912), they undergo development in definite stages in the lymphocyte, and ultimately become free-swimming spirochæte-like bodies in the blood-plasma. Having assisted in the investigation of the development of these so-called Kurloff's bodies, I was struck with the similarity between them and the new structures seen in the epithelial cells of the seminal vesicles of the earth-worm, and the matter was further investigated. I am now convinced that these structures in the earth-worm also represent phases in the life-cycle of a new parasite which is very similar in its developmental figures to *Lymphocytozoon cobayæ*.

The cells in the worm which contain these peculiar structures are the large cœlomic epithelial cells which form the walls of the vesiculæ seminales of *Lumbricus terrestris*. The body first observed was a large spherical sac containing a closely woven skein, staining deep blue with azur stain, lying embedded within the cytoplasm of the epithelial cell. The method of examination employed was that devised by H. C. Ross (1910) for the examination of blood-cells *in vitro*, and it is known as the "jelly method." A 2-per-cent. solution of agar in distilled water is boiled, sterilised, and filtered. To 5 c.c. of the filtrate is added 1 c.c. of a 1-per-cent. solution of azur II, and the total bulk of the mixture is made up with water to 10 c.c. in a test-tube. When molten, a small quantity of the jelly is allowed to spread itself in a thin film on a microscope slide, where it will set when it cools. A drop of a suspension of the cells of the seminal vesicles in citrated normal saline solution is placed on a cover-glass, which is then inverted and allowed to fall flat on to the set jelly film. The spermatozoa, the nuclei of the epithelial cells and of the seminal cells all stain deeply. Within the epithelial cells the parasites also stain in sharp contrast with the surrounding cytoplasm, so that their morphology can be readily determined. The first stage of the parasite within the cell consists of a blue dot or granule situated in what looks like a vacuole which colours only faintly (Plate 14, fig. 1). In the next stage observed, the dot has apparently divided, for the vacuole or sac now contains several blue granules (fig. 2). The dots then elongate into rods (fig. 3), and each rod becomes lengthened and splits longitudinally (fig. 4), until the parasite appears like a bunch of painted sticks—a sheaf embedded within the cytoplasm of the epithelial cell, its host. This process of splitting continues and at length there is a series of spiral coils wound up within the sac, which is still enveloped by the protoplasm of the epithelial cell (figs. 6 and 7).

In some specimens observed by the jelly method, the parasite is separated from its host-cell, and is found stained and lying free as in fig. 5. The final stage is that of a free spirochæte (fig. 8). This is the fully developed parasite, having a long slender body, pointed at one end and blunt at the other. It is nearly always found coiled up, but occasionally it is almost straight. Its protoplasm is homogeneous, and contains at irregular intervals in its length small chromatin masses, varying in number from five to twelve, which are characteristic of spirochætes. These spirochætes have not only been seen free, but also swimming actively in the seminal fluid when the latter has been examined by dark ground illumination. They are readily differentiated from spermatozoa by their length, granulation, and peculiar movements, nor can there be any confusion between this parasite and the well-known *Monocystis lumbrici* or the spermatophores. So far as I can ascertain, its existence has not been recorded before. It is interesting to note, however, that intracellular phases of spirochætes have been described in other animals by A. Balfour, H. B. Fantham, E. Hindle, Sir William Leishman, and others. Its resemblance to the parasite of the guinea-pig is very remarkable; the earth-worm parasite is larger, however; develops in the fixed epithelial cells of the vesiculæ seminales; and has not been seen in the blood of its host nor in the cœlomic fluid, but it passes through corresponding phases. The parasite is present in about one earth-worm out of five.

Summary.

A description of "bodies" found within some of the epithelial cells of the vesiculæ seminales of the earth-worm. They closely resemble "Kurloff's bodies"

found within the lymphocytes of guinea-pigs, and by means of the jelly method of examination, the development of these bodies into free spirochætes is demonstrated in the same way that it has recently been shown that "Kurloff's bodies" also become spirochætes. I would suggest that these new parasites be called *Spirochæta lumbrici.*

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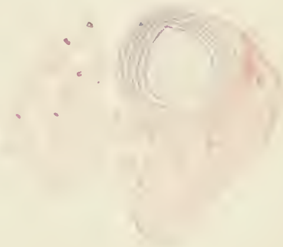

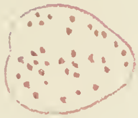

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VI. THE NITROGENOUS CONSTITUENTS OF LIME-JUICE.

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(Preliminary Communication.)

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(Received November 22nd, 1912.)

In connection with the work previously published on beri-beri [Funk, 1911, 1912, 1], in which the physiological importance of certain nitrogenous substances, belonging most probably to the pyrimidine group, was demonstrated, a similar fractionation of lime-juice was undertaken for the investigation of scurvy.

From the classical work of Holst and Fröhlich [1907, 1912] on scurvy we know that this disease has a close analogy to beri-beri and is also due to a lack of some substance in the food. They specially advanced our knowledge of this disease by inducing in animals (rabbits and guinea-pigs) a disease which possesses a great similarity in symptoms to human scurvy. They produced the experimental scurvy by an exclusive diet of oats or autoclaved vegetables and were able to prevent it by an addition to this diet of fresh vegetable-juice, unboiled milk or lime-juice. Lime-juice itself has been known for many years as an excellent remedy for scurvy.

Some time ago I was able to show [1912, 2] that lime-juice contains an anti-neuritic substance curing polyneuritis in birds, produced by an exclusive diet of polished rice, and a substance, present in another fraction, which is only capable of prolonging the life of these birds. Further experiments have shown that pyrimidine substances in general possess this life-prolonging action and a paper on this subject will shortly appear. No anti-scorbutic substance however was obtained from lime-juice.

I had at first intended to try each fraction, obtained during this investigation, on guinea-pigs fed on oats, but in this I found considerable difficulties. The animals refused all food after a period of three weeks and even an addition of freshly made potato-juice or lime-juice was not able to save them from starvation. The same negative result was obtained with fractions of lime-juice which might have been expected to contain the anti-scorbutic substance in a more concentrated form.

The only positive results were obtained with milk, 50 c.c. daily of unboiled milk in addition to the oats diet preventing loss of weight and onset of scurvy. Even after the elimination of caseinogen and the other proteins present in milk, the latter keeps its preventive power. In doing this extreme care must be taken not to destroy the substance, and all boiling must be carefully avoided. The caseinogen is precipitated by addition of acetic acid at 50^c and lactalbumin by dialysed iron-solution, kaolin however carries the substance down. The solution is then concentrated by freezing it and taking the supernatant liquid. This subject is being further investigated. This unstable character of the anti-scorbutic substance renders it unlikely that the usual method of separation, with the unavoidable use of alkali, would lead to its isolation. Although no anti-scorbutic substance was obtained from lime-juice, several new compounds have been detected and are described below.

Besides a substance, which apparently belongs to the terpene group and which has only been analysed and not further investigated, as having no direct connection with the subject, three nitrogenous substances have been isolated. One belongs to the purine group, the second to the pyrimidine group and the third to the choline group. The small yield obtained did not allow however of a complete investigation, so that this paper must be regarded as preliminary.

EXPERIMENTAL.

407 litres of commercial lime-juice were precipitated in portions of 15 litres each with 2800 grms. of neutral lead acetate. The bulky precipitate was filtered off on a large Buchner funnel and the residue pressed out in a hydraulic press. The combined filtrates were freed from the excess of lead, first by an addition of sulphuric acid, then by sulphuretted hydrogen, and were then concentrated *in vacuo* at low temperature. During the distillation crystals separated out, which were filtered off, and weighed when dry 258 grms. These were recrystallised from 20 $^0/_0$ acetic acid and 162 grms. were obtained in needles. The substance proved to be free from nitrogen and was recrystallised for analysis once more from water. It is

nearly insoluble in all other solvents, except acetic acid, and is volatile with steam; M.P. 97°–100° (uncorr.).

0·1357 grm. gave	0·3400 CO_2 and	0·1312 H_2O.
Found	68·33 % C;	10·73 % H.
Calc. for $C_{13}H_{24}O_3$;	68·42 % C;	10·52 % H.

This substance probably belongs to the terpene group; it possesses a very characteristic agreeable smell when heated. The nature of this compound, which has no direct relationship with the scurvy problem, was not further investigated.

To the filtrate from this substance enough sulphuric acid was added to make a 5 % solution and then a 50 % solution of phospho-tungstic acid until precipitation was complete. The precipitate obtained amounted to nearly 12 kg., and consisted chiefly of potassium salt.

INVESTIGATION OF THE PHOSPHO-TUNGSTATE PRECIPITATE.

The precipitate was decomposed in portions of 1 kg. each with 2½ kg. baryta in a mortar and the mixture was shaken on the shaking machine for one hour. The precipitate was then filtered off, suspended in water and again shaken. The excess of baryta was eliminated from the combined filtrates by the careful addition of dilute sulphuric acid. The solution obtained was then neutralised with nitric acid, as it was strongly alkaline, and evaporated *in vacuo* to about two litres. In this solution the usual fractionation with silver nitrate was performed and three fractions were obtained. The first fraction came down on addition of a silver nitrate solution and contained the substances of the purine group. This precipitate was filtered off and to the filtrate a saturated solution of silver nitrate was added until a drop of the liquid gave with a cold baryta solution a brown precipitate which indicates an excess of silver. A saturated aqueous baryta solution was then added until a drop of the clear liquid gave only a small precipitate with silver nitrate and ammonia. The second precipitate consists of pyrimidine bases and substances of the histidine type. To the filtrate pulverised baryta was added until the whole of the silver was precipitated. The fraction thus obtained contains, in addition to traces of pyrimidine substances, compounds of the arginine group. The last filtrate contains substances of the choline group. All the fractions were investigated separately.

INVESTIGATION OF THE FIRST SILVER FRACTION.

The bulky precipitate obtained by a simple addition of silver nitrate was filtered off, well washed with water, suspended in water and the double silver nitrate salts converted into the silver salts by heating them on the water-bath with ammonia. The precipitate was filtered off and washed with water, until the filtrate was free from nitric acid. It was then suspended in water and decomposed with sulphuretted hydrogen. The solution on evaporation yielded 1 grm. of substance, which however contained a little ash. It was recrystallised from water and gave 0·7 grm. of crystals in the form of plates. After drying *in vacuo* at 110° they became brown at 240° and melted at 282° (corr.). The substance is precipitated by mercuric acetate and partially by gold chloride. It differs from the known purine bodies by the great solubility in hot water and the absence of all known reactions for these derivatives. Heated with nitric acid it becomes yellow, and brown when more strongly heated, no change of colouration being produced on the addition of alkali.

0·3855 grm. loses at 110° (*vacuo*) 0·0190 grm. H_2O
0·1176 ,, requires 32·7 c.c. N/10 H_2SO_4
0·1372 ,, ,, 38 c.c. ,, ,,
0·1226 ,, gave 0·1788 CO_2 and 0·0196 H_2O

Calc. for $C_6H_7O_2N_5$ (Mol. wt. = 181)		Found	
C	39·78 %	39·77	
H	3·86 %	3·55	
N	38·67 %	38·77	38·92
½ Mol. H_2O	4·73 %	4·92	

INVESTIGATION OF THE SECOND SILVER FRACTION.

The second silver nitrate precipitate was filtered off, washed well with water and decomposed with sulphuretted hydrogen. In the filtrate the last traces of baryta were taken out with a very dilute solution of sulphuric acid and the solution was evaporated *in vacuo.* As the residue did not deposit any crystals, the solution was precipitated with mercuric sulphate. The solution obtained by decomposition of the precipitate showed no tendency to crystallisation, but on addition of a picric acid solution an oil separated, which was decanted and dissolved in acetone. After evaporation a solid powder was obtained which amounted to 0·35 grm. This was recrystallised from a mixture of alcohol and acetone and yielded about 0·1 grm. of brown prisms, which decomposed at 205°–210°. An amount of this fraction corresponding to 0·01 grm. of the picrate was decomposed and given to a polyneuritic pigeon which showed a considerable improvement and died only after four days.

The mercuric sulphate filtrate, freed from mercury and sulphuric acid, was evaporated *in vacuo* and the residue left in a desiccator after addition of alcohol. After a few days a colourless substance separated out which amounted to 0·7 grm. After recrystallisation from dilute alcohol 0·25 grm. was obtained in the form of microscopical spherolites, which after drying at 110° *in vacuo*, melted and decomposed at 188°–189°.

0·0958 grm. subst. required 7·5 c.c. N/10 H_2SO_4; 10·96 % N.
0·1034 ,, gave 0·1625 CO_2 and 0·0636 H_2O; 42·86 % C; 6·83 % H.
Calc. for $C_9H_{18}O_6N_2$ (Mol. wt. = 250) 43·2 % C; 7·2 % H and 11·2 % N.

The third silver fraction yielded but little substance. On evaporation of the solution obtained by decomposing the precipitate no crystalline compound separated out. Picric acid solution however gave a precipitate which after standing formed crystalline, yellow spherolites, which began to decompose at 260°; yield 0·1 gr.

INVESTIGATION OF THE SILVER NITRATE FILTRATE.

Of this filtrate, which amounted to 4600 c.c., only 220 c.c. were taken for further investigation. To this liquid after elimination of silver and baryta, phospho-tungstic acid was added and 420 grms. of a dry precipitate were obtained, which was decomposed in the ordinary way. The solution of the bases belonging to the choline group was neutralised with hydrochloric acid and evaporated *in vacuo* to dryness. To get rid of the last traces of water the residue was redissolved in alcohol and the alcohol evaporated *in vacuo*. Finally an alcoholic solution was made and an alcoholic solution of sublimate was added as long as a precipitate was formed. This was recrystallised from hot water with the addition of a little sublimate. It was found to be very slightly soluble in water. In this way 10·5 grms. of crystals were obtained in the form of cubes. These were redissolved in water and decomposed with sulphuretted hydrogen. The filtrate was evaporated *in vacuo* to dryness, the residue dissolved in alcohol and evaporated again. The alcoholic solution finally obtained was precipitated with an alcoholic platinic chloride solution, 1·3 grms. of a pale yellow precipitate being obtained which was recrystallised from dilute alcohol; 0·85 grm. was thus obtained in the form of needles, which, after drying at 110° *in vacuo*, melted at 220° (uncorr.). The substance gave the following figures on analysis, after being dried *in vacuo* at 110°.

0·1666 grm. gave 0·0451 grm. Pt
0·1622 ,, required 4·2 c.c. N/10 H_2SO_4
0·2173 ,, gave 0·2104 CO_2; 0·0878 H_2O; 0·0589 Pt and 0·2586 AgCl.

Calc. for $(C_8H_{15}O_2NHCl)_2PtCl_4$ (Mol. wt. 724).	Found:
C : 26·52 ;	26·40
H : 4·42 ;	4·48
N : 3·86	3·62
Pt : 26·94 ;	27·07 ; 27·1
Cl : 29·55	29·44

On igniting the platinum salt a peculiar smell was noticed, which was quite different from that given by choline.

A substance of this formula, with similar properties (M.P. 219°), was described by v. Braun [1908] as the platinichloride of methylpiperidylacetic betaine. The substance is being further investigated.

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XXI. AN ATTEMPT TO ESTIMATE THE VITAMINE-FRACTION IN MILK.

By CASIMIR FUNK, *Beit Memorial Research Fellow.*
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(*Received February 17th, 1913.*)

The isolation of the beri-beri vitamine from milk [Funk, 1912, 1], together with the important fact observed by Andrews [1912], that infantile beri-beri occurs when the children are fed by mothers suffering from beri-beri, suggest a new line of investigation into the etiology of infantile scurvy and rickets. The problems which present themselves in connection with these investigations have been already set forth [Funk, 1912, 2] and will be fully treated in an account in the *Ergebnisse der Physiologie*, Vol. 13. These problems are briefly the following. (1) What is the normal amount of vitamines in milk of different species, including the human? (2) Is there a definite relationship between the amount of vitamines secreted in the milk and that ingested in the food? This point is of special interest because of the periodical appearance of rickets in winter and disappearance in summer, a period when the cows return to pasture. (3) What effect have boiling and pasteurisation on the vitamine content of milk? All these points await their solution until a method for determining such small quantities is available. The present inquiry shows that the ordinary chemical methods for estimating vitamines can hardly suffice and attention at present must therefore be directed to colorimetric methods.

Nevertheless the results described below give an idea of the amount of vitamines possibly present in milk. The whole vitamine fraction (nitrogen precipitated by phosphotungstic acid in the alcoholic extract of the dried milk) from 1 litre of milk amounts to 1–2·5 mgr. nitrogen. This would correspond to 1–3 cg. vitamine ($C_{17}H_{20}O_7N_2$). After the elimination of the vitamine-fraction the residual nitrogen amounts to 20–50 mgr. per litre milk. This residual nitrogen represents in all probability allantoin; assuming this to be correct, one litre of milk contains 0·06–0·15 grm. allantoin, a figure in good agreement with that obtained by Ackroyd [1911] by means of a direct method, namely 0·199 grm.

The results further indicate that for a chemical investigation of the vitamines of milk it will be necessary to start on a large amount of milk. At the same time they show that the artificial protein-free milk, such as was used by Osborne and Mendel [1912] in their experiments on growth, differs from true protein-free milk in lacking these nitrogenous substances. It is therefore quite conceivable, as the experiments of Hopkins and Neville [1913] point out, that these substances play an important part in the process of growth.

As the figures below show, the milk after removal of fat by centrifuging has lost about 50 % of vitamine and allantoin.

The milks used in these experiments were from London dairies. For each experiment a litre was evaporated in vacuo at 30°, using a distilling flask constructed of two parts from which therefore the residue could be quantitatively removed. The process of drying the residue was hastened in the last stages by an addition of alcohol. The residue was powdered in a mortar and dried in a vacuum desiccator to constant weight. The fine powder was then shaken with a definite amount of alcohol (400 c.c.) in a shaking machine for two hours. The extract was filtered and of the filtrate an aliquot part was evaporated in vacuo. The residue was extracted with water and the extract transferred to a measuring flask. The watery extract was filtered and the filtrate found in every case to be entirely free from proteins. A measured quantity was acidified slightly with sulphuric acid and precipitated with 10 % phosphotungstic acid. After standing for 48 hours the precipitate was filtered and washed with dilute sulphuric acid. In accordance with my previous results this phosphotungstic acid precipitate can be regarded as the vitamine-fraction. Both in the precipitate and filtrate the nitrogen was estimated by Kjeldahl, N/20 normal solutions being used for titration.

		Weight of residue	Nitrogen of the vitamine fraction in mgr. (phosphotungstic acid precipitate)	Residual nitrogen in mgr. (phosphotungstic acid filtrate)
1.	Centrifuged milk	100	2·54	50·9
	Control	99·5	2·46	49·6
2.	Uncentrif.	133	1·7	30·7
	Centrif.	98	1·53	16·8
3.	Uncentrif.	127	2·5	22·7
	Centrif.	90	1·4	12·1
4.	Uncentrif.	129	1·96	29·6
	Centrif.	92	1·2	15·3
5.	Uncentrif.	132	2·4	49·2
	Centrif.	96	1·6	27·3
6.	Centrif.	89	1·06	22·4
7.	Centrif.	105	1·83	44·9

In this connection the behaviour of allantoin when boiled with Ruhemann's reagent (triketohydrindene hydrate) should be noted. According to Abderhalden and Schmidt [1911] allantoin when boiled for one minute with Ruhemann's reagent does not give any colouration. But although this is so, if the boiling be continued for 2–3 minutes a violet colouration appears and this reaction might be used as a test for allantoin. Since the crude product obtained by oxidation of uric acid by permanganate gave the reaction at once, probably due to admixture with allanturic acid, and allantoin is known to be destroyed by boiling, it was thought that a decomposition product of allantoin, probably allanturic acid, was the cause of this reaction. A sample of pure allantoin was therefore boiled with PbO_2 and another with water. Both samples gave a strong allanturic acid reaction after a certain time of boiling, the sample with PbO_2 giving however the stronger reaction. The reaction seemed to disappear after prolonged boiling. The reaction may also be obtained from hydantoin.

It is interesting to note, that we have in allantoin an example of a substance, which like vitamine is destroyed by boiling.

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THE PREPARATION FROM YEAST AND CERTAIN FOODSTUFFS OF THE SUBSTANCE THE DEFICIENCY OF WHICH IN DIET OCCASIONS POLYNEURITIS IN BIRDS. By CASIMIR FUNK.

(*From the Bio-chemical Department, Lister Institute of Preventive Medicine.*)

THIS substance has recently been isolated(1) in what appears to be pure condition from rice-polishings. It crystallises in colourless needles which melt at 233° and the results of the single analysis, which the amount of material at my disposal permitted, indicated the formula $C_{17}H_{20}N_2O_7$. The administration of this substance (about 0·02 grm.) to pigeons suffering from polyneuritis effected a rapid cure. The small yield obtained did not however allow of many such curing experiments being performed and as the substance was not recrystallised, doubts about its purity might be entertained. A confirmation of these facts was therefore absolutely necessary and in the first instance yeast which is known to be curative was chosen as the source of the material since it promised to give a better yield than rice-polishings. It was of great interest to see whether yeast contained the same substance as rice-polishings or only an analogous compound. The yield obtained did not however come up to expectation.

In spite of the facts that a few grams of pressed yeast are sufficient to cure a pigeon suffering from polyneuritis and, as Eykman recently showed(2), the curative substance can be extracted by means of alcohol, the yield was even smaller than in the case of rice-polishings. Before Eykman's paper appeared the author tried to extract yeast with alcohol but only a small part of the active substance could thus be removed. The bulk of the substance remained in the yeast, even when the latter was boiled with alcohol for several hours. This suggests that the substance is to a great extent present in the yeast in a combined state. Schaumann(3) in a recent publication has confirmed the results previously published by the author, but thinks that besides the curative substance another substance is required. He founds this opinion upon the ground that the yield of curative substance obtained from yeast or rice-polishings compared with the quantity of original yeast or polishings necessary for cure is so exceedingly small. As just stated however the extraction is far from being complete, and during the fractionation the yield of the curative substance becomes smaller and smaller.

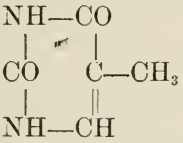
The yeast was worked up by the two following different methods. In the first case the alcoholic extract was simply evaporated and the residue hydrolysed with sulphuric acid, the solution being then treated as in the case of rice-polishings (1). By precipitating first with phosphotungstic acid and then with silver nitrate and baryta, half a gram of the curative substance was obtained.

In the second case the alcoholic extract of yeast after evaporation was extracted with water. On precipitation with silver nitrate and baryta however only a minute quantity of the curative substance could be detected. The filtrate from the silver precipitate contained the bulk of it and it was necessary to hydrolyse this with acid before the precipitation could be effected. The resulting fraction unfortunately contained all the pyramidine bases which are known to be constituents of yeast nucleic acid and of these uracil and thymine could be isolated in a pure state. From this mixture it was found that the reagents ordinarily employed for the separation of the curative substance also precipitated more or less of the pyramidine bases. After a long and troublesome fractionation however a small quantity of the pure curative substance was obtained which showed the characteristic properties.

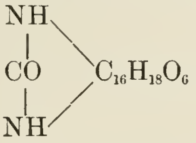
A large number of cures was effected on pigeons and there can be no doubt that this substance is the sole curative agent, the dose necessary being 2–4 cgr., smaller doses were not tried.

Nature and mode of combination of the curative substance. In the first place it is necessary to correct the previous statement that the curative substance was isolated in form of a nitrate. This is not the case, the substance which was analysed having been found to be the free base. In the meantime (4) I have suggested the name *vitamine* for it as being one of those nitrogenous substances, minute quantities of which are essential in the diet of birds, man and some other animals. The curative substance must be regarded as a base, probably belonging to the pyramidine group. The aqueous solution is of neutral reaction and does not react with acids. By boiling with copper oxide no copper salt is formed and therefore it is not an aminoacid. Recrystallised from dilute alcohol the substance melts at 233°, at the same temperature as the curative substance from rice. It gives the same reactions and both substances must therefore be considered as identical. It is precipitated in a pure state by mercuric acetate as well as by silver nitrate, but not by mercuric sulphate or nitrate.

All these properties suggest that the curative substance is a pyramidine base, analogous to uracil and thymine and that it probably is a constituent of nucleic acid. On this view the two nitrogens would be combined as in other pyramidine bases to form a ureid.



Thymine



Vitamine.

Only a constitution of this kind would explain the neutral reaction of the substance and the great analogy to the other pyramidine bases.

The curative substance was also isolated by analogous methods from milk (this fact being very important for our knowledge of infantile scurvy) and brain. Everything suggests that in all these cases the curative substance is identically the same. Further, a substance curing avian polyneuritis was found in lime-juice and is at present being more closely investigated.

EXPERIMENTAL.

Investigation of yeast.

Extraction of yeast followed by hydrolysis of the extract. 75 kg. of air-dried and pulverised yeast were extracted in portions of $2\frac{1}{2}$ kg. each with 4 litres alcohol on the shaking machine for 2 hours. The yeast was then filtered off and the filtrate evaporated in vacuo. The residue, corresponding to $12\frac{1}{2}$ kg. of yeast in each case, was hydrolysed with 1 litre of 10 °/₀ sulphuric acid for 5 hours. The fatty acids were filtered off and the filtrate diluted with water to obtain a 5 °/₀ solution of sulphuric acid, and precipitated with phosphotungstic acid. In this way 927 grm. of dry phosphotungstate were obtained from 75 kg. of yeast. The precipitate was decomposed with 2500 grm. of baryta in the usual way. In the filtrate the excess of baryta was eliminated carefully with sulphuric acid and the filtrate, after neutralisation with nitric acid, was evaporated in vacuo to a volume of 1 litre. To the liquid a silver nitrate solution was added until a drop gave with a cold solution of baryta a brown precipitate of Ag_2O. A bulky precipitate, consisting of purine bases, separated which was filtered and to the filtrate pulverised baryta was added until a drop of the solution gave with silver nitrate and ammonia only a trace of a white precipitate. The precipitate thus formed was filtered off, thoroughly washed with water and decomposed with H_2S. From the filtrate the last traces of baryta were eliminated carefully with a very dilute solution of sulphuric acid. Alcohol was added and the solution evaporated in a vacuum desiccator, 0·6 grm. of a crystalline substance being obtained which was recrystallised from hot dilute alcohol. On cooling 0·45 grm. of colourless needles separated which melted after drying at 233°, the same melting point as that of the substance from rice-polishings. The substance was precipitated by mercuric acetate but not by mercuric nitrate and sulphate. When its solution was boiled with cupric oxide no copper salt was formed. The substance gives no precipitate with nitron and cannot therefore be a nitrate. Seven pigeons were cured with this substance, 2–4 cgr. being employed. The filtrate from the silver nitrate precipitate, freed from silver and baryta, was ineffective in curing pigeons.

Extraction of yeast followed by exhaustion of the extract with water. The alcoholic extract from the second lot of yeast (100 kg.), obtained as above, was extracted on a water bath with water, filtered and the aqueous solution precipitated with phosphotungstic acid in 5 % sulphuric acid solution. In this way 2800 grm. of a dry phosphotungstate were obtained which were decomposed with 5500 grm. of baryta. The filtrate freed from an excess of baryta, was neutralised with HNO_3 and evaporated in vacuo. The residue was precipitated with silver nitrate in the manner previously described, and the fraction obtained with $AgNO_3$ and baryta decomposed, alcohol added and the solution evaporated in a vacuum desiccator. In a few days 2·1 grm. of crystals separated which presented however a different appearance from the substance previously obtained and which were less soluble and could therefore be recrystallised from water. 1·8 grm. of substance were obtained on cooling which crystallised in form of rosettes. The substance, after drying at 110° in vacuo, melted at 330° (corr.).

·1507 substance gave ·489 CO_2 and ·0238 H_2O; C : 43·09; H : 3·60.

·2054 (by Kjeldahl's method) required 36·4 c.c. N/10 H_2SO_4; N : 24·81.

These figures correspond to uracil which has been found in the autolysis products of the yeast nucleic acid.

Calculated for $C_4H_4N_2O_2$; C : 42·82; H : 3·59; N : 25·05.

The filtrate from the uracil was hydrolysed with 5 % sulphuric acid for 5 hours. As uracil is precipitated by mercuric nitrate whilst the curative substance is not, mercuric nitrate was added and 24·8 grm. of dried mercury salt were obtained. In both precipitate and filtrate from the precipitation with $Hg(NO_3)_2$ only traces of the curative substance could be detected.

Investigation of the silver nitrate filtrate. Although in the case of the hydrolysed extract the silver nitrate filtrate was found to be completely inactive, this filtrate in the second case was found to be strongly active. It was thought therefore that the curative substance being in combination did not come down with silver nitrate. The filtrate freed from silver and baryta was precipitated again with phosphotungstic acid. In this way 820 grm. of precipitate were obtained which were decomposed with 2000 grm. of baryta. The filtrate was made up to 5 °/₀ sulphuric acid and hydrolysed for 5 hours. After a careful elimination of sulphuric acid the liquid was concentrated in vacuo to about 200 c.c. and precipitated with mercuric sulphate. After standing for 24 hours the liquid was filtered off, the precipitate well washed and decomposed with H_2S. The filtrate from the HgS was freed from H_2SO_4 by means of baryta and was concentrated in vacuo. The mercuric sulphate however also carries down the bulk of the curative substance. Besides uracil 0·25 grm. thymine could be isolated in this fraction. It was obtained in form of plates which melted at above 300° and gave the following figures on analysis:

·1609 substance (by Kjeldahl's method) required 25·6 c.c. N/10 H_2SO_4. Found 22·27 °/₀ N, $C_5H_6O_2N_2$ requires 22·22 °/₀ N.

The filtrate from the mercuric sulphate, treated with silver nitrate and baryta, gave a silver salt which after decomposing yielded a small quantity of crystals (0·05 gr.), which melted at 233° and possessed curative properties. Thus a separation of the curative substance was effected, even in this case.

Isolation of allantoin from rice-polishings.

51 kg. rice-polishings were treated in portions $1\frac{1}{2}$ kg. with 4 litres of absolute alcohol on the shaking machine for 1 hour. The polishings were filtered off and the alcoholic extract corresponding to $10\frac{1}{2}$ kg. of polishings in each case was melted on the water bath with 1 litre of water and the aqueous extract was evaporated in vacuo. The aqueous solution was precipitated with basic lead acetate and the filtrate, freed from lead, was precipitated with silver nitrate and baryta. The silver salt formed was filtered off, well washed and decomposed with H_2S. The filtrate

from the Ag_2S was freed from the last traces of baryta by an addition of very dilute sulphuric acid and the final filtrate evaporated in vacuo to a small volume and the residue mixed with alcohol and transferred into a vacuum desiccator. In a few days the residue began to crystallise. The crystals (yield 0·6 grm.) were filtered off and recrystallised from 5 % hydrogen peroxide. The substance melted with decomposition at 231°. When mixed with synthetical allantoin the melting point was not depressed.

0·1282 substance by Kjeldahl's method required 32·4 c.c. N/10 H_2SO_4. Found 35·39 % N; calculated for allantoin $C_4H_6N_4O_3$ 35·49 % N.

The substance was entirely different from the curative substance. When mixed with an equal weight of the latter the melting point was depressed 21°. Allantoin is not precipitated by phosphotungstic acid but is precipitated by mercuric sulphate. These properties can be used for the separation of the two substances. Synthetical allantoin (prepared from uric acid) given to seven pigeons suffering from polyneuritis did not cure them, but seemed to prolong the life of the birds. This point however requires further investigation.

Detection of the curative substance in other foodstuffs.

Milk. 1398 grm. of a commercial dried milk preparation known under the name of 'Trumilk,' was extracted with 3 litres of alcohol on the shaking machine for 12 hours. The residue was filtered off and extracted in a Soxhlet apparatus with ether for 12 hours. The combined alcohol and ether extracts were evaporated in vacuo and the residue hydrolysed with 2 litres of 10 % H_2SO_4 for 5 hours. On cooling the solid fatty acids were separated off and the aqueous extract was precipitated with phosphotungstic acid. The precipitate was washed with 5 % H_2SO_4 and when dry weighed 51 grm. This was decomposed with 125 grm. of baryta and the liquid freed from baryta was then found to be effective in curing pigeons suffering from polyneuritis. After the solution had been neutralised with HNO_3, $AgNO_3$ was added, the precipitate formed was separated and to the filtrate baryta was added. The precipitate obtained was filtered and decomposed with H_2S. By evaporating this solution in a vacuum desiccator a small quantity of a crystalline substance in the form of needles was obtained which melted at 230° and possessed the characteristic curative properties.

Ox-brain. 2180 grm. of dried ox-brain were shaken out with 4 litres of alcohol. This was filtered off and the residue extracted again with ether and finally with alcohol. The extracts were evaporated to dryness in vacuo and hydrolysed with 10 % H_2SO_4 for 5 hours. The solution gave on precipitation with phosphotungstic acid 220 grm. of a dry precipitate which was decomposed in the usual way with 600 grm. of baryta. In the filtrate the excess of baryta was eliminated with weak H_2SO_4 and the filtrate, neutralised with HNO_3, was evaporated in vacuo to about 100 c.c. This solution was found to be curative and was treated with $AgNO_3$ and baryta as before. The silver fraction after decomposition yielded on evaporation a little of a crystalline substance, which was apparently not quite pure, it melted at about 203° but possessed the curative properties.

Lime-juice. 42 litres of commercial lime-juice (nitrogen content 0·35 %) were made up to the content of 5 % H_2SO_4 and were precipitated with a solution of phosphotungstic acid. The precipitate, which weighed when dry 1200 grm., was treated in a mortar with 2500 grm. of baryta, shaken on the shaking machine, filtered off, suspended in water and shaken again. In the combined filtrates the excess of baryta was removed with dilute H_2SO_4. The filtrate from the $BaSO_4$ was neutralised with HNO_3 and the solution, which was found to be curative, was evaporated in vacuo. After the precipitation of the purine bases with $AgNO_3$, a saturated solution of baryta was added to the filtrate so long as the fluid gave a white precipitate with $AgNO_3$ and ammonia. The silver precipitate obtained in this way amounted to 5·9 grm.; it was decomposed with H_2S and the solution evaporated in a desiccator. No appreciable quantity of crystals separated out but the solution was found to be curative for pigeons. Further investigation of this material is in progress.

SUMMARY.

1. The substance preventing beri-beri was isolated from a number of foodstuffs and the previous statements of the author fully confirmed.

2. The base obtained from all these foodstuffs seems to be the same. For curing pigeons (*per os*) 0·02–0·04 grm. were employed.

3. The chemical properties of the curative substance suggest that it is a pyramidine base, forming a constituent of a nucleic acid.

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FURTHER EXPERIMENTAL STUDIES ON BERI-BERI. THE ACTION OF CERTAIN PURINE- AND PYRIMIDINE-DERIVATIVES.

BY CASIMIR FUNK, *Beit Memorial Research Fellow.*

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IN one of my earlier papers on this subject(1), I was able to show, that a substance may be isolated from rice-polishings, which administered to birds, suffering from polyneuritis, produces a complete recovery.

The same substance has been isolated by analogous methods from yeast, milk and brain(2). The chemical properties of the substance suggested to me, that it probably belongs to the pyrimidine-group. As the substance is very unstable, an attempt was made to reduce the manipulations to the strict minimum and to avoid the action of alkali, which seems to have a very destructive effect. It was tried to simplify the method and to make the yield better, but all methods failed and I have to use again my former method. An extraction on a large scale is now in progress. I have also repeated the experiments of Suzuki, Shimamura and Odake(3) and I have found, that the method does not work in our conditions. With Malay polishings, stored very likely for a considerable time before their arrival in this country, I was completely unsuccessful.

It was thought, for instance, that the process could be shortened, by leaving out the precipitation with phospho-tungstic acid. A fractionation of rice-polishings, carried out on these lines, did not yield the beri-beri-vitamine[1], but a substance of similar properties, namely allantoin, which in the first case passes into the filtrate[2]. This substance was also tested on neuritic birds and was found not to be curative, but only to effect some improvement and to retard the death of the animals for several days. These results together with the probability of a pyrimidine structure for the beri-beri-vitamine, led me to try the effect of known purine- and pyrimidine-bodies on neuritis in birds. These experiments had two purposes, first to see, whether these substances have a similar action to allantoin, the second whether the animal is able to use them for building up the necessary "vitamine." Besides this this investigation had to help the chemical investigation of the anti-neuritic substance, which has great difficulties because of the small yield.

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[1] This name being suggested for the anti-neuritic substance for the present (4), till the chemical structure is found out.

[2] Schaumann in a recent paper (*Arch. f. Schiffs- und Tropenhygiene* XVI. p. 825. 1912) claims to have isolated the auti-neuritic substance before me. The substance was not identified and the method used in his case could only lead to the isolation of allantoin, which, as I have shown, is present in rice-polishings in larger proportion, than the beri-beri-vitamine.

The experiments which will be described in this paper show beyond any doubt, that a number of purine- and pyrimidine-substances possess a very marked action on neuritic birds. Although the sick birds rarely die later than 24 hours after the onset of the symptoms, as a number of controls have shown, I was able by addition of these substances to keep them alive for 4, 5 and often more days. The daily supply of 30 grm. rice with an addition of allantoin (synthetical product 0·025 grm. a day) delays the onset of symptoms for several days and lessens the waste of weight. The birds die with very slight or even without any neuritic symptoms. Similar to allantoin is the action of hydantoin especially as concerns influence on weight. Some substances have even a very marked effect on the symptoms, on several occasions results were obtained which could be regarded as real cures and lasted for 4–5 days. I propose to prepare some of these substances synthetically and to add them daily to the polished rice diet. Only a few of these substances used in these experiments were prepared by me. Most of them were supplied by Dr Levene, Professor Winterstein and Professor Schittenhelm, and I would like to express here my gratitude for their kindness. My explanation of the action of these substances is, that owing to a similar structure, they are able to replace some of the physiological functions of the beri-beri-vitamine.

They explain at the same time the action of nucleic acids on neuritic birds and also the action of certain alcoholic extracts (phosphatide fraction), which when free from the vitamine, contain as I have shown, a large amount of nucleic acid derivatives as impurity.

As uric acid has no effect on pigeons, it is made probable, that birds are not able to form allantoin from uric acid, a result which is in concordance with the fact, that their organs were found free from uricolytic ferment. In this respect the birds seem to show a great analogy to man. As Ackroyd(5) shewed, the amount of allantoin present in the food largely accounts for the amount of this substance secreted in the human urine.

The pigeons were fed as usual on polished rice; after the onset of symptoms a dose stated each time (as a rule 0·1 grm.), dissolved or in suspension in water was introduced in the crop and the animals closely observed. In other experiments (with allantoin and hydantoin) the substance was added daily and the animals weighed regularly, until they died.

Substance	Dose (grm.)	No. of animals	Origin of the preparation	Results	
Uric acid ...	0·2	2	Kahlbaum	None.	
Adenin ...	0·1	2	Winterstein	Survival 30–50 hours.	
Guanin ...	0·1	2	Levene	Practically none.	
Hypoxanthin ...	0·1	2	Winterstein	3–5 days survive.	
Xanthin ...	0·1	2	,,	2–4 ,, ,,	
Paraxanthin ...	0·1	4	,,	Cure and survival for 6 days, in other 3 cases no marked effect.	
Uracil	0·1	2	Own prep.	Improvement of the symptoms and survival 3–4 days.	
Thymin ...	0·05	2	Levene	None.	
Allantoin ...	0·1	7	Own prep.	2–3 days	Very marked improvement in state of the animals.
Hydantoin ...	0·2	5	,,	2–9 days	
Yeastnucleic acid ...	1	1	Böhringer	4 days survival.	
				Survival	
Thymusnucleic acid	0·2	2	Schittenhelm	9–14 days	Very marked improvement.
Guanosin ...	0·1	3	Levene	1–4 ,,	
Adenosin ...	0·05	1	,,	4 ,,	
Cytidin	0·05 of the nitrate	2	,,	2–6 ,,	

Duration experiments.

Allantoin experiment.

	30 grm. of rice+0·025 grm. allantoin daily			Controls, 30 grm. of rice		
Initial weight	I 379	II 322	III 357	IV 395	V 305	VI 319
4 days	406	315	372	361	298	312
8 ,,	363	276	338	323	258	288
12 ,,	317	263	322	294	244	240
16 ,,	303	256	308	276	226	—
18 ,,	↓	↓	↓	↓	190	
19 ,,				232	—	—
20 ,,	296	246	292	—	—	—
24 ,,	296	247	—	—	—	—
25 ,,	272	242	—	—	—	—
% Loss of weight	28·3	24·8	18·2	41·2	37·7	24·7

From this experiment we see, that the birds with allantoin lose less in weight, show slighter symptoms. The birds with allantoin lose 1 grm. per 100 grm. weight, whereas those without 2·1 grm. daily.

Hydantoin experiment.

	Pigeons on 30 grm. of rice and 0·1 grm. hydantoin daily			Controls on 30 grm. daily		
Initial weight	I 367	II 371	III 406	IV 421	V 367	VI 385
After 2 days	365	391	413	416	360	384
4 ,,	357	404	420	437	368	399
6 ,,	351	384	424	403	352	395
8 ,,	343	376	404	391	336	376
10 ,,	327	364	398	378	313	356
12 ,,	330	350	389	365	305	355
13 ,,	312	↓	↓	↓	280	↓
14 ,,	—	340	375	348	—	340
16 ,,	—	336	368	340	—	327
18 ,,	—	337	352	341	—	313
19 ,,	—	305	↓	↓	—	↓
20 ,,	—	—	354	342	—	308
22 ,,	—	—	341	339	—	299
23 ,,	—	—	↓	300	—	↓
24 ,,	—	—	344	—	—	308
25 ,,	—	—	↓	—	—	266
26 ,,	—	—	339	—	—	—
28 ,,	—	—	—	—	—	—
30 ,,	—	—	291	—	—	—
$^0/_0$ Loss of weight	14·9	17·7	28·4	29·0	21·5	30·9

Whereas the controls lost 1·33 grm. daily from 100 grm. weight, the birds with hydantoin lost only 0·98 grm.

CONCLUSIONS.

1. Certain purine- and pyrimidine-substances have a very marked effect on pigeons suffering from polyneuritis; a relationship between the chemical structure and the action could not however be established.

2. The action of allantoin suggests, that pigeons are not able to convert uric acid into allantoin.

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STUDIES ON BERI-BERI.

FURTHER FACTS CONCERNING THE CHEMISTRY OF THE VITAMINE-FRACTION FROM YEAST.

By CASIMIR FUNK,
BEIT MEMORIAL RESEARCH FELLOW.
(From the Biochemical Department, Lister Institute.)

At the end of 1911[1] I described a method for the isolation of the vitamine-fraction from rice polishings. At this time only a single substance was detected, which was studied to some extent in its chemical properties and curative power. Early in 1912,[2] I was able to show that the same method might be successfully used for the isolation of the vitamine-fraction from other foodstuffs, namely, milk, yeast, brain, and lime juice. Even at this time it was noticed that the isolated pure substance of the melting point 233° had a slightly diminished curative power as compared with the crude product. As a second substance at that time could not be isolated, there was no explanation of this fact available. Since then I have been able to show[3] that a number of purin and pyrimidin derivatives possess a slight curative effect—a very interesting fact, which, however, did not simplify the problem.

It was therefore conceivable that besides the substance of the melting point 233°, small quantities of other substances might be present in this fraction, the physiological effect of which therefore would be the resultant of the individual actions of these constituents.

A fractionation of the yeast extract was therefore carried out on a large scale and the results of this are shortly summarized in this preliminary communication.

The alcoholic extract of 100 kg. dried yeast was prepared, following the method already described in my earlier papers. The fractionation itself was carried out without any change of methods and only the silver and baryta fraction which represents the vitamine-fraction was carefully investigated. The total amount of substances obtained in this fraction from 100 kg. of dried yeast was 2.5 grams. This initial product was crystalline and melted at 210° (uncorrected).

A number of pigeons were treated with this product. Polyneuritis was induced in them by artificial feeding with an exclusive diet of polished rice. After a time, varying from ten to twenty-one days, they developed the characteristic symptoms of avian polyneuritis, as they are represented in the figures 1, 3, and 5. By a series of controls it was found[3] that the animals in this condition live rarely longer than six to twelve hours, twenty-four hours being perhaps the utmost limit. The product mentioned above was injected into the pectoral muscle in quantities varying from 4 to 8 mg. and in each case a complete recovery was obtained (Figs. 2, 4, 6). Before and after treatment the animals were kept on an unchanged diet of polished rice. In spite of that they lived after the recovery for four to six days without any symptoms of neuritis. As the food did not secure a new supply of vitamine, the birds developed neuritis again after the injected substance was apparently used up for metabolic processes. In one of these cases after a new supply of substance a second cure was effected.

CASE I.—Severe symptoms of polyneuritis; 4 mg. of the above-mentioned product were injected into the pectoral muscle. Three hours later a great improvement; next day a complete recovery; four days later shows again symptoms, and dies.

CASE II.—Severe symptoms (Fig. 1);* 8 mg. injected. Two hours later a complete recovery (Fig. 2); was able to fly away, and had to be photographed, therefore, in a cage; relapsed after six days, and was again cured by a new injection of the product.

CASE III.—Severe symptoms (Fig. 3); injected 4 mg. Recovery after three hours (Fig. 4); lived for four days.

CASE IV.—Polyneuritis (Fig. 5); injected 8 mg. After three hours a complete recovery (Fig. 6), which lasted for four days.

We see from these experiments that the initial vitamine-fraction possesses the undiminished curative power of the original yeast extract. As in some cases the change of diet alone (an addition of maize, for instance) is capable of improving very markedly the condition of the sick birds, the fact that an unchanged diet of polished rice was kept throughout the experiment cannot be too much emphasized.

By a further fractionation I was able to separate the crude fraction into at least three substances, each of

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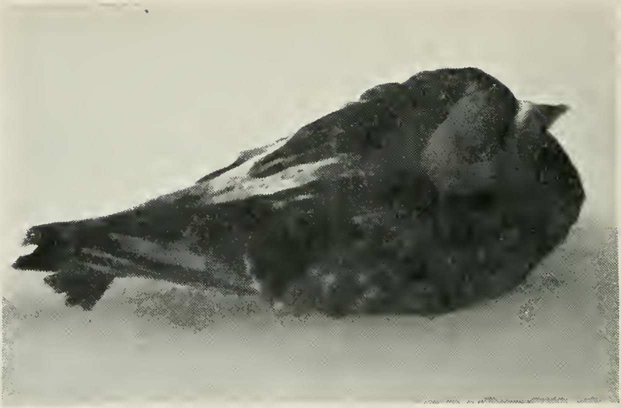

FIG. 1.

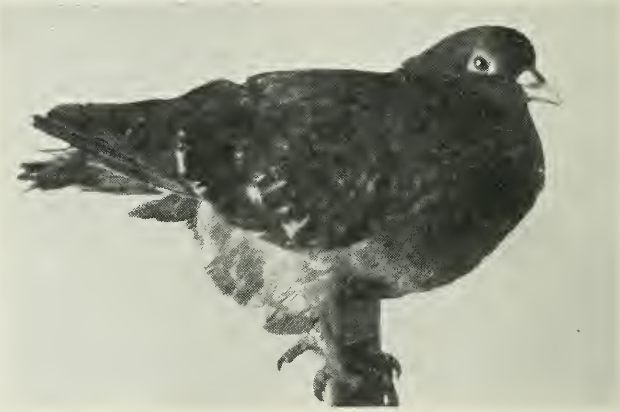

FIG. 2.

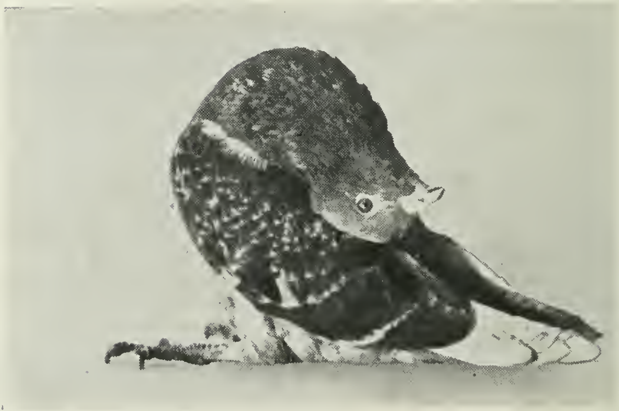

FIG. 3.

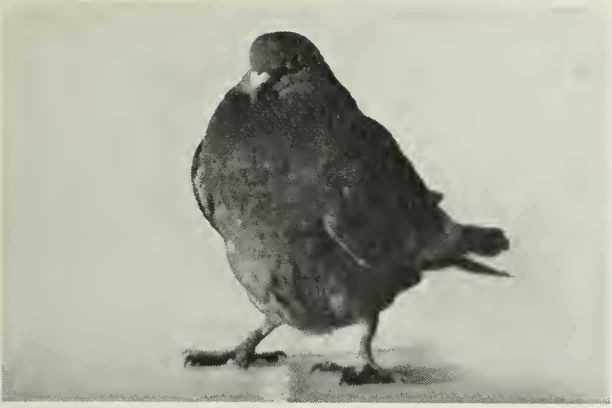

FIG. 4.

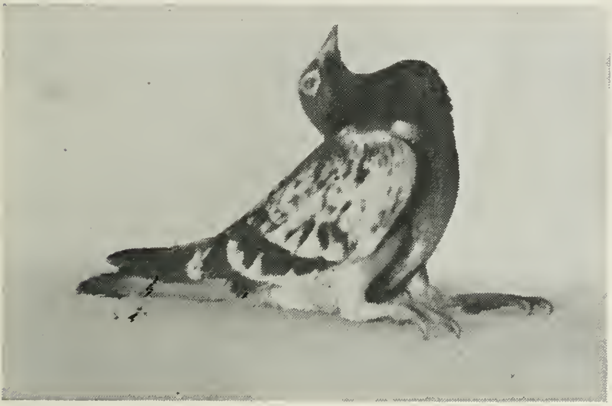

FIG. 5.

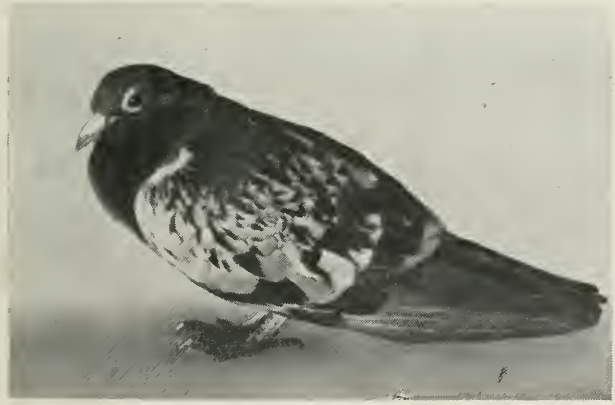

FIG. 6.

which has been carefully purified (recrystallization till constant melting point) and analysed. The original product (2.5 grams) yielded the following substances:

Substance I.—1.6 grams, melting at 229° (corrected); needles and prisms nearly insoluble in cold water. When mixed with the substance previously obtained from yeast[2] it does not show any depression of melting point; the substances might therefore be regarded as identical.

Substance II.—0.4 gram, very soluble in cold water, and gives a difficultly soluble picrate. The substance melts at 235° (uncorrected).

Substance III.—0.3 gram, more soluble in water than the first substance; melts at 222° (uncorrected).

These observations have an interesting parallel with the case of pituitary extract, as recently recorded by Fühner,[4] where also in the first instance a crystalline fraction was isolated, which showed the whole physiological action of the gland, but on further fractionation was found to consist of not less than four different substances. Each of these had a specific action, but the effect of the original product could only be obtained by the collective action of all the four substances. It will be interesting to see whether the same is the case with the vitamine-fraction, or whether a single substance plays a preponderating part in the process of cure.

For therapeutic purposes, therefore, the best results may at present be expected by a paraenteral administration of the whole vitamine-fraction, especially in acute cases which have proceeded too far to be influenced by a change of diet—in such cases, for example, as have been described by Strong and Crowell[5] and Schüffner.[6] This fraction which is suggested to be employed contains the active constituents unimpaired of the original starting material, and is free from any harmful substances.

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* The photographs have been kindly made for me by Dr. A. B. Macallum, jun., to whom I would like here to express my thanks.

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XXII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEW). I. CARBOXYLASE.

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(*Received February 17th, 1913.*)

When zymin is thoroughly washed with water a residue is left, which no longer has the power of fermenting glucose, but regains this power when the washings are added to it, even when the latter have been boiled [Harden and Young, 1910]. The dried yeast used by Lebedew behaves in a precisely similar manner [Euler and Bäckström, 1912].

It is therefore a matter of considerable interest to ascertain how far this treatment affects the various enzymes which are known to exist in zymin and dried yeast. It can thus be ascertained whether these enzymes are themselves soluble and whether if insoluble they require for their action the presence of a soluble substance of the nature of a coenzyme.

It is also probable that some light may be thrown on the possible function of some of these enzymes in the process of alcoholic fermentation.

Carboxylase.

Since the discovery by Neuberg and Hildesheimer [1911] of the unexpected fact that yeast, yeast juice and zymin readily and rapidly decompose pyruvic acid and other α-ketonic acids with evolution of carbon dioxide and formation of an aldehyde, the opinion has been expressed in many quarters that pyruvic acid may form a stage in the enzymatic decomposition of glucose into alcohol and carbon dioxide [Neubauer and Fromherz, 1911; Neuberg and Kerb, 1912; Kostytschew, 1912; Lebedew 1912].

On the other hand it is possible that carboxylase is quite independent of the enzymes of alcoholic fermentation, its function being that of decomposing the α-ketonic acids formed by the deaminisation of the α-amino-acids (see Neubauer and Fromherz, 1911).

In order to examine the action of washed yeast preparations on pyruvic acid, experiments were made not only on the free acid but also on the sodium salt in presence of weak inorganic acids, by the aid of which the acidity was diminished, whilst at the same time the whole or almost the whole of the carbon dioxide produced was evolved.

The evolution of carbon dioxide was observed by the aid of the apparatus previously described [Harden, Thompson and Young, 1910]. No quantitative estimations of acetaldehyde were made, but its presence was proved by the reaction with Schiff's reagent in all cases in which an evolution of carbon dioxide was observed. Control experiments were at the same time carried out with glucose and phosphate. It was thus found that the residue obtained by washing zymin and dried yeast (Lebedew) until they could no longer ferment glucose, was capable of decomposing pyruvic acid quite readily. The interesting fact is thus ascertained that carboxylase does not require the presence of a coenzyme removable by washing in order to exert its characteristic reaction on pyruvic acid. This result does not however allow any definite conclusion to be drawn as to the possible function of carboxylase in alcoholic fermentation. It can only be concluded that if the decomposition of pyruvic acid actually be a stage in the alcoholic fermentation of glucose, the soluble coenzyme is required for some change precedent to this, so that in its absence the production of pyruvic acid cannot be effected. The following experiments illustrate the results obtained. The solutions throughout were saturated with carbon dioxide at the temperature of the bath before the commencement of incubation.

Exp. 1. A sample of Schroder's dried yeast (nach Lebedew) was washed 3 times on the centrifuge with water and then brought into acetone and dried. The following experiments were then made.

(*a*) 2 g. washed and acetoned yeast + 10 c.c. 1 per cent. pyruvic acid + 40 c.c. H_2O.

(*b*) 2 g. washed and acetoned yeast + 25 c.c. 1 per cent. pyruvic acid + 25 c.c. H_2O.

(*c*) 2 g. washed and acetoned yeast + 25 c.c. 1 per cent. pyruvic acid + 5 c.c. 0·3 molar Na_2HPO_4 + 20 c.c. H_2O.

(*d*) 2 g. washed and acetoned yeast + 0 pyruvic acid + 5 c.c. 0·3 molar Na_2HPO_4 + 45 c.c. H_2O + 2 g. glucose.

These were incubated at 25° and the evolution of CO_2 measured.

Time	(*a*)	(*b*)	(*c*)	(*d*)
1 hr. 50 mins.	3·5	1·6	18·6	1·9

The action of the washed yeast on glucose was extremely small (*d*) and very little fermentation of the free pyruvic acid (*a* and *b*) occurred. In presence of sodium phosphate however a considerable evolution of CO_2

occurred (18·6 c.c.) and a strong reaction for acetaldehyde was given by the filtrate. The small effect on free pyruvic acid is ascribed to the effect of the acidity of the solution on the enzyme; in presence of sodium phosphate, on the other hand, the acidity is mainly due to NaH_2PO_4 formed by interaction of the pyruvic acid with the Na_2HPO_4.

Exp. 2. A similar experiment was made with similar results using the same washed and acetoned yeast, but washing it again 3 times to remove the last trace of coenzyme.

10 g. of acetoned yeast were washed and made up to 85 c.c.

(*a*) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. H_2O.

(*b*) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. 0·3 molar Na_2HPO_4 (approx. equivalent to the pyruvic acid).

(*c*) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 molar Na_2HPO_4 + 2 g. glucose.

Time	(*a*)	(*b*)	(*c*)
55 mins.	4·1	22·0	1·2 c.c.

Here again the action on the free acid is small, whereas a considerable evolution of CO_2 occurs in presence of phosphate. The amount produced is really greater than that evolved since when pyruvic acid and sodium phosphate are present in equivalent amount a portion of the CO_2 is retained as $NaHCO_3$ in equilibrium with NaH_2PO_4.

$$CH_3 \cdot CO \cdot COOH + Na_2HPO_4 \rightarrow CH_3 \cdot CO \cdot COONa + NaH_2PO_4 \rightarrow$$
$$CH_3 \cdot CHO + NaHCO_3 + NaH_2PO_4 \rightleftarrows CH_3 \cdot CHO + Na_2HPO_4 + CO_2 + H_2O.$$

Accordingly, the combined CO_2 was determined after incubation and, in a separate sample, before incubation and it was thus found that 12·9 c.c. of CO_2 were retained and should be added to the amount observed in (*b*), making a total production of 34·9 c.c.

Exp. 3. The effects of neutral potassium citrate and of excess of free boric acid in presence of the sodium salt of pyruvic acid were tried. The yeast employed was a similar preparation to that used in Exp. 2.

(*a*) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. 0·3 M. potassium citrate neutral to phenolphthalein.

(*b*) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 M. sodium phosphate + 2 g. glucose.

(*c*) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 M.K. citrate.

(*d*) ,, ,, + 21 c.c. 1 per cent. pyruvic acid + 2·4 c.c. N. KHO + 3 g. H_3BO_3.

Time	(*a*)	(*b*)	(*c*)	(*d*)
1 hr. 25 mins.	4·5	1·3	1·0	25·6

The sample of yeast had practically no action on glucose (*b*), comparatively little on pyruvic acid in presence of citrate (*a*) and a large action in presence of free boric acid (*d*). This result therefore is in agreement with the idea that the action of the enzyme is greatly inhibited by acid, citric acid being much stronger than boric acid.

Exp. 4. 10 g. dried yeast was washed 3 times and made to 100 c.c.

(*a*) 25 c.c. yeast suspension + 25 c.c. 1 per cent. pyruvic acid + 2·5 c.c. N. KHO + 3 g. boric acid + toluene.

(*b*) 25 c.c. yeast suspension + 21 c.c. H_2O + 5 c.c. 0·3 M. Na_2HPO_4 + 2 g. glucose + toluene.

Time	(*a*)	(*b*)
1 hour	21·04	0·2 c.c.

Here again a good fermentation of pyruvic acid is produced by a sample of washed yeast incapable of fermenting glucose, the total evolution from which in 18 hours was only 0·4 c.c.

Exp. 5. 20 g. zymin (Schroder) were washed 3 times and made to 100 c.c.

(*a*) 25 c.c. yeast suspension + 22·5 c.c. H_2O + 2 g. glucose + 5 c.c. 0·3 M. Na_2HPO_4.

(*b*) „ „ + 25 c.c. 1 per cent. pyruvic acid + 2·5 c.c. N. KHO + 3 g. boric acid.

Time	(*a*)	(*b*)
43 mins.	17·8	0·8
18 hours	22·7	0·8

Washed zymin therefore has the same action as washed dried yeast.

SUMMARY.

Zymin and dried yeast (Lebedew) after being freed from coenzyme by washing and thus rendered incapable of fermenting glucose, readily decompose pyruvic acid into carbon dioxide and acetaldehyde, provided that the acidity of the solution is kept low.

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The Chemical Action on Glucose of a Variety of Bacillus coli communis (*Escherich*), *obtained by Cultivation in Presence of a Chloroacetate.* (*Preliminary Notice.*)

By A. HARDEN, F.R.S., and W. J. PENFOLD.

(Received May 18,—Read June 20, 1912.)

(Biochemical Department of the Lister Institute.)

Penfold has shown (1) that when *Bacillus coli communis* (Escherich) is plated out on nutrient agar containing 0·5 per cent. sodium chloroacetate, a large number of small colonies and a few large ones are produced. The cultures obtained from some, but not all, of the large colonies were found to have lost the power of producing gas from glucose when grown in glucose peptone water in a test tube provided with a Durham gas tube. Under the same conditions these same organisms still produced gas from mannitol, but in diminished proportion. Several other intestinal organisms have since been found by Harden to behave in a similar manner on chloroacetate agar, whereas *B. lactis aërogenes*, which produces a different type of decomposition of sugar, has hitherto proved resistant to this method of selection. In order to ascertain what other modification the chemical action of the original strain had undergone, comparative quantitative experiments were carried out with the original and the selected organisms.

Product.	Percentage of sugar used.		Carbon atoms per molecule of glucose.		Harden's theory for *B. coli communis*. Carbon atoms.
	Normal *B. coli.*	Selected *B. coli.*	Normal *B. coli.*	Selected *B. coli.*	
Alcohol	17·22	5·5	1·35	0·43	1
Acetic acid	20·60	10·02	1·24	0·60	1
Formic acid	2·55	3·53	0·10 } 0·81	0·14 } 0·22	1
Carbon dioxide	17·30	2·00	0·71	0·08	
Lactic acid	40·60	77·6	2·43	4·66	3
Succinic acid	4·80	None	0·29	None	
	93·07	98·65	6·12	5·91	6
H (as gas) c.c. per grm. of sugar	80·6	12·6			
H as gas + H equivalent of formic acid	92·9	29·8			

The medium employed in both cases contained 10 grm. peptone and 20 grm. glucose per litre, 10 grm. of chalk being added per litre. The flasks were connected with the gas-collecting apparatus described by Harden, Thompson, and Young (2), and the air of the flask replaced by nitrogen before incubation The estimations were made as previously described (3). The results of two such experiments are given in the table on p. 415, the products being expressed both in percentage of the sugar used and as the number of carbon atoms of the glucose molecule to which they correspond.

These results must be regarded as preliminary, but are of sufficient interest to justify some remark at the present stage of the work.

In the first place, it is to be noted that, although the selected organism gave no gas at all when tested by the Durham tube method, it yielded about 12·6 c.c. of hydrogen per gramme of sugar when grown anaërobically in presence of chalk as against 80·6 given by the original organism. Similarly the amount of gaseous CO_2 formed from the sugar was found to be 10·3 c.c. per gramme, but as this number is the difference of two large volumes (the total gaseous CO_2 + CO_2 dissolved in the medium − CO_2 liberated by the acids formed) no great accuracy attaches to it. The reason of this different behaviour is not understood and is at present being investigated.

Apart from this, an examination of the results shows that the main difference between the actions of the two bacteria is to be found in the increased proportion of lactic acid, and the correspondingly diminished proportion of the other products, formed by the selected organism.

Harden (3) has previously shown that the action of organisms closely allied to *B. coli communis* is roughly represented by the equation

$$(1) \quad 2C_6H_{12}O_6 + H_2O = 2C_3H_6O_3 + C_2H_6O + C_2H_4O_2 + 2CO_2 + 2H_2,$$

which requires the proportions of products shown in the last column of the table. Actually there is always less CO_2 than this produced, and with the strain of normal *B. coli communis* here employed there is also somewhat more alcohol and acetic acid and less lactic acid formed. The relations between the products of the selected organism appear, however, to be quite incompatible with this equation.

The explanation which suggests itself is that the products observed as the result of action of the normal organism are formed by three independent enzymes. One of these converts sugar into lactic acid:

$$(2) \quad C_6H_{12}O_6 = 2C_3H_6O_3.$$

The other probably produces alcohol, acetic acid, and formic acid, and the last of these is in all probability decomposed by a third enzyme into carbon dioxide and hydrogen:

$$(3)\quad C_6H_{12}O_6 + H_2O = C_2H_4O_2 + C_2H_6O + 2HCO_2H$$
$$= C_2H_4O_2 + C_2H_6O + 2CO_2 + 2H_2.$$

These enzymes may occur in different proportions in different individual organisms, and the process of selection would then result in the survival of an organism containing a large proportion of the lactic acid enzyme and a small proportion of that producing alcohol, acetic acid, and formic acid. It may be noted that the selected organism still retains the power of decomposing formic acid into CO_2 and H_2, and, moreover, its products do not contain more of this acid than those of the normal bacillus. The absence of gas formation, therefore, is not due to an accumulation of formic acid. It is thus seen that the change produced in the organism does not really cause its chemical action to approximate to that of *B. typhosus*, an organism which also yields no gas, but differs from *B. coli* by producing a large amount of formic acid.

The foregoing numbers are not sufficiently accurate to decide with certainty whether the relative proportions of alcohol, acetic acid, and formic acid produced by the selected organism are the same as those formed by the original bacillus. Taking the alcohol as standard, the carbon as formic acid (including gaseous CO_2) should be 0·27, instead of 0·22 observed, and that as acetic acid 0·41 instead of 0·6. The deviation in the last case may be due to the difficulty of effecting the quantitative separation of acetic acid from a large proportion of lactic acid.

Further experiments may be expected to throw light on these points.

If, however, the foregoing explanation be found correct, the study of the chemical action of these selected organisms should form a valuable means of investigation into the nature of the various processes of fermentation effected by bacteria, and the subject is being prosecuted from this point of view.

REFERENCES.

(1) Penfold, 'Roy. Soc. Med. Proc.,' 1911, p. 97.
(2) 'Biochemical Journal,' 1910, vol. 5, p. 230.
(3) Harden, 'Chem. Soc. Journ.,' 1901, p. 610.

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The Action of Enzymes on Hexosephosphate.

By VICTOR J. HARDING.

(Communicated by A. Harden, F.R.S. Received May 21,—Read June 20, 1912.)

(Biochemical Department of the Lister Institute.)

According to the theory advanced by Harden and Young,* the presence of phosphate is essential for the alcoholic fermentation of sugar by yeast-juice. In the presence of the fermenting complex, the phosphate and sugar react together, with the simultaneous production of equivalent quantities of carbon dioxide, alcohol, and hexosephosphate. The hexosephosphate is then hydrolysed by an enzyme present in yeast-juice, with formation of a hexose and free phosphate, and the latter then again undergoes the first reaction with more sugar. The phosphate thus repeatedly passes through a cycle which may be represented by the following equations:—

$$(1) \quad 2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O,$$

$$(2) \quad C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4.$$

The normal rate of fermentation of excess of sugar by active yeast-juice is therefore dependent upon the rate at which phosphate is set free from the hexosephosphate, and any acceleration of this reaction would increase the rate of fermentation of the sugar. Thus the addition to yeast-juice and sugar of a hexosephosphatase, that is, an enzyme capable of hydrolysing hexosephosphate, would be expected to bring about this result, and the following experiments on the action of various enzyme preparations on sodium hexosephosphate have been carried out primarily with the object of finding such an enzyme.

As a result, it was found that enzymes of very different origin (Ricinus lipase and almond emulsin) were capable of hydrolysing hexosephosphate, but the effect of adding these to fermenting yeast-juice has not yet been ascertained.

Preparation of Hexosephosphoric Acid.—The solution of hexosephosphoric acid used in these experiments was prepared as described by Young,† by decomposing the lead salt with hydrogen sulphide. The solution of free hexosephosphoric acid was made neutral to litmus by a known volume of sodium hydrate solution, and such a neutral solution was used in all the experiments. The amount of hexosephosphoric acid was previously determined by titration of 1 c.c. with N/10 KOH, phenolphthalein as indicator. In all experiments the term hexosephosphate is used to designate the solution neutral to litmus. The extent of hydrolysis was in each case ascertained by precipitating the liberated phosphate with magnesium citrate mixture, igniting and weighing as magnesium pyrophosphate. All results, as well as the concentration of the original hexosephosphate solutions, are expressed in terms of magnesium pyrophosphate.

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* 'Roy. Soc. Proc.,' 1908, B, vol. 80, p. 299.

† 'Roy. Soc. Proc.,' 1909, B, vol. 81, p. 531.

The following enzyme preparations were employed:—

(*a*) lipase from castor oil seeds; (*b*) autolysed ox pancreas; (*c*) emulsin; (*d*) zymin extract; (*e*) autolysed yeast-juice.

(*a*) *Lipase from Castor Oil Seeds* (Ricinus communis).—This was prepared by extracting the freshly ground seeds of *Ricinus communis* with ether until fat free, and using the residue as the lipase preparation. It was found to be highly active.

The results of three experiments are given in the accompanying table, which refers in each case to the following set of mixtures:—

A. 0·3 grm. lipase + 3 c.c. N/5 acetic acid + 15 c.c. water.
B. „ „ „ + 15 c.c. hexosephosphate.
C. Same as B, but boiled before incubation.

The experiments were carried out at 25° in presence of toluene (0·5 c.c.), and different hexosephosphate solutions were employed in each set of experiments.

No.	Original hexosephosphate as $Mg_2P_2O_7$.	Time of incubation in hours.	Free phosphate, grm. $Mg_2P_2O_7$.			Phosphate liberated, B − C.
			A.	B.	C.	
1	0·5835	24	0	0·1259	0·0428	0·0831
		48	0	0·1836	0·0320	0·1516
2	0·5665	120	0	0·2746	0·0416	0·2330
3	0·6274	240	0	0·3655	0·0359	0·3296

It is seen that the preparation had a considerable hydrolytic action on the hexosephosphate.

(*b*) *Ox Pancreas.*—The fresh ox pancreas, freed as far as possible from connective tissue, was allowed to undergo autolysis for four days under toluene at 37°. The liquid was then filtered through a coarse muslin cloth to remove any undigested pancreas, and the filtrate used in the experiments.

The following mixtures were incubated for 48 hours at 37° in presence of toluene, and the free phosphate estimated as before:—

A. 5 c.c. autolysed ox pancreas + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. The same as B, but the pancreas mixture employed was boiled.

Original hexosephosphate = 0·5351 grm. $Mg_2P_2O_7$.
Free phosphate in A ...... = 0·0 „
„ B ...... = 0·0915 „
„ C ...... = 0·0814 „
Phosphate liberated ...... = 0·0101 „

Practically no action had taken place.

(*c*) *Emulsin.*—The emulsin was prepared as described by H. E. and E. F. Armstrong and Horton.*

The experiments were carried out at 37° in presence of toluene, the following mixtures being employed:—

A. 10 c.c. emulsin solution + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. Same as B, but boiled emulsin employed.

Original hexosephosphate = 0·6274 grm. $Mg_2P_2O_7$.

	After	
	48 hours.	144 hours.
Free phosphate in A	0·0	0·0
„ „ B	0·1750	0·3064
„ „ C	0·0342	0·0563
Phosphate set free by enzyme ...	0·1408	0·2501

Emulsin has, therefore, a considerable hydrolytic action on hexosephosphate.

(*d*) *Zymin Extract.*—10 grm. of "zymin" preparation were digested four days with 30 c.c. water at 25°. The resulting mixture was filtered and used in the experiment. It was found to contain considerable amounts of phosphate.

An aqueous extract of zymin (yeast treated with acetone) was found only to have a slight action on hexosephosphate. This is seen from the following experiment carried out at 25° in presence of toluene, the mixture being incubated for three days:—

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* 'Roy. Soc. Proc.,' 1908, B, vol. 80, p. 324.

A. 5 c.c. zymin extract + 15 c.c. water.
B. ,, ,, + 15 c.c. hexosephosphate.
C. Same as B, but zymin extract boiled.

Original hexosephosphate............... = 0·6274 grm. $Mg_2P_2O_7$.
After incubation, free phosphate in—
A = 0·0625 ,,
B = 0·1618 ,,
C = 0·0910 ,,
Phosphate set free by enzyme......... = 0·0708 ,,

(*e*) *Autolysed Yeast-juice.*—A sample of yeast-juice which had been allowed to remain at 0° for a fortnight was used. It was found to be incapable of fermenting glucose.

The following mixtures were incubated at 25° for 24 hours in presence of toluene, and the free phosphate estimated:—

A. Autolysed yeast-juice 25 c.c. + 10 c.c. water.
B. ,, ,, + 10 c.c. hexosephosphate.
C. As B, but boiled yeast-juice employed.

Original hexosephosphate .... = 0·3776 grm. $Mg_2P_2O_7$.
After incubation, free phosphate in—
A = 0·1389 ,,
B = 0·2232 ,,
C = 0·1409 ,,
Phosphate set free by enzyme......... = 0·0823 ,,

Hexosephosphate is thus hydrolysed to some extent by autolysed yeast-juice. This is in agreement with with the experiments of Harden and Young, who showed that in actively fermenting yeast-juice most of the phosphate was present in the combined form, but was gradually set free by an enzyme hexosephosphatase, after the juice had lost its fermentative activity.*

An attempt was made to ascertain if hexosephosphatase could be isolated from the autolysed yeast-juice: 50 c.c. of autolysed yeast-juice were added with constant stirring to a mixture of 400 c.c. alcohol and 400 c.c. ether. The white precipitate was filtered immediately at the pump, washed once with ether, and air dried. A very small amount of a brownish sticky precipitate remained. This was dissolved in 50 c.c. of water and allowed to stand overnight with a little toluene. It was then filtered through muslin, and the solution thus obtained examined as to its hydrolytic properties towards hexosphosphate.

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* 'Roy. Soc. Proc.,' 1908, B, vol. 80, p. 299.

The mixtures A, B, C were incubated with toluene at 25° for 24 hours:—

A. 10 c.c. above solution + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. As B, but enzyme solution previously boiled.

Original hexosephosphate .... = 0·5665 grm. $Mg_2P_2O_7$.
After incubation, free phosphate in—
A = 0·0494 „
B = 0·1268 „
C = 0·0755 „
Phosphate set free by enzyme .... = 0·0513 „

The hexosephosphatase, therefore, can be precipitated by means of alcohol and ether.

Summary.

(*a*) Ricinus lipase and emulsin possess a slow hydrolytic action on hexosephosphate.

(*b*) Autolysed ox pancreas is almost without action upon hexosephosphate.

(*c*) "Zymin" extract hydrolyses hexosephosphate slowly.

(*d*) Autolysed yeast-juice possesses a marked action on hexosephosphate.

(*c*) An alcohol-ether precipitation of autolysed yeast-juice possesses considerable activity towards hexosephosphate.

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On some Parasites of the Scoter Duck (*Œdemia nigra*), and their Relation to the Pearl-inducing Trematode in the Edible Mussel (*Mytilus edulis*). By H. LYSTER JAMESON, M.A., D.Sc., Ph.D., and WILLIAM NICOLL, M.A., D.Sc., M.D.

(Text-figures 11 & 12.)

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Of the many questions connected with the formation of pearls in the common Edible Mussel (*Mytilus edulis*) the identity and life-history of the pearl-inducing organism is one of the most important. It was shown ten years ago by Jameson (1902) that the agent in this particular case is the larva of a parasitic Trematode, which, instead of secreting a cyst of its own, as is usual with such larvæ, stimulates the mussel to form around it a sac of epidermal cells. These cells possess the same physiological properties as the outer shell-secreting epidermis, and eventually, on the death of the Trematode larva, secrete conchyolin and calcareous salts, which, deposited in concentric layers around the remains of the worm, become the pearl. Attempts were made by Jameson to trace the life-history of this parasite, but the difficulties in the way of working out the complete life-cycle of digenetic Trematodes are considerable, and the results obtained by him in 1902 have not been accepted as entirely conclusive.

With regard to the parasite in *Mytilus*, the two main questions to be solved were: (1) Whence does it come? and (2) Whither does it go? From a consideration of Trematode development in general, it was obvious that these larvæ in *Mytilus* must have passed a previous stage, as a sporocyst or redia, in some other molluscan host, and it was equally obvious that they were destined to become adult in some final vertebrate host, to be sought among the animals that eat the mussel.

With regard to the earlier part of the life-history, Jameson was struck by the occurrence in *Tapes decussatus*, which lives associated with the pearl-bearing mussels in the harbour of Billiers (Morbihan), Brittany, of sporocysts containing *Cercariæ* closely resembling those in *Mytilus* and differing from them chiefly in size. Jameson subsequently found similar sporocysts in a different situation in the common Cockle (*Cardium edule*) on the pearl-bearing mussel-beds at Piel, Lancashire, and these have since been rediscovered by Lebour (1906) and Nicoll (1906). The occurrence of these sporocysts in *Cardium* is of particular interest in view of the fact that the cockle, as a rule, lives in close association with the mussel and is on that account a not unlikely intermediate host for the mussel parasite.

Herdman (1903–6) failed to find this parasite in Piel cockles, and seems to have doubted Jameson's assertion that it occurred there; but we have found it over and over again in cockles both from the original station at Piel, and from the cockle-bed at Foulney in the same neighbourhood.

Infection-experiments were undertaken by Jameson in 1901, in order to prove the transference of the parasite from *Tapes* to *Mytilus*, but although he claimed success in these experiments, they are still open to the objection that some at least of the mussels used were already infected before the experiment was undertaken *.

Pending the results of further experiments, there are only the structural characters of the two larval forms to go upon. Morphologically there is a very close agreement between the cercaria in *Cardium* and that in *Mytilus*. They both agree in having the oral sucker not more than one-fifth of the body-length, and in its being not more than one-third as large again as the ventral sucker. In both the pharynx is comparatively large. The differences which exist are small. For instance, the oral sucker in the *Mytilus* cercaria is relatively larger than that in the *Cardium* cercaria; the ventral sucker is larger in the former in proportion to the oral and it is slightly nearer the posterior end of the body instead of, as might have been expected, further forward. Again, the œsophagus is shorter and the diverticula longer. These discrepancies, although minute, are sufficient to give pause in too hastily concluding that the two forms belong to one and the same species.

With regard to the final host of this parasite, there can be little doubt but that Jameson was right in supposing it to be one of the mussel-eating ducks, such as the Scoter and the Eider; but the form he figured as the adult in 1902 from the Common Scoter, which he referred to *Gymnophallus* (*Lecithodendrium*) *somateriæ*, and which we are now describing below under the name *G. œdemiæ*, is certainly not the adult of the larva which occurs in *Mytilus*, but is a much smaller species *.

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* [I am at present repeating these experiments, with the aid of a Government Grant from the Royal Society.—H. L. J.]

In order to reconcile the small size of the larvæ which he found in the Scoter at Billiers in 1902 with the larger dimensions of the larvæ in the mussel, Jameson was obliged to have recourse to an unlikely hypothesis. This was commented on by Odhner, who suggested that Jameson had probably encountered a mixture of two species, one probably being *G. bursicola* and the other a new species of the same genus. As the present investigation will show, the latter part of Odhner's supposition was correct: while, with regard to the former, until further specimens from Billiers can be examined, it is impossible to say whether the larger examples observed by Jameson at that station were *G. bursicola* or *G. dapsilis*.

Since the publication of Jameson's paper, two species, namely *Gymnophallus bursicola* Odhner and *G. dapsilis* Nicoll, have been suggested as the possible adult form. Before the discovery of the second of these species Odhner (1904) gave it as his opinion that the probable adult was *G. bursicola*, a parasite of the Bursa Fabricii of the Eider Duck (*Somateria mollissima*). At the same time he stated that a larva identical with that in *Mytilus* occurs in *Saxicava rugosa* in the Arctic regions. At that time there could be little question as to the probable correctness of his view, for no other known species was so likely to be the adult of the parasite in *Mytilus*. The discovery by Nicoll (1907) in the Scoters of a second bursicolous *Gymnophallus* closely resembling, yet distinct from, *G. bursicola*, showed that Odhner's conclusions could not be accepted without further study, for *G. dapsilis* appears to have even stronger claims than *G. bursicola* to be considered the adult of the larva in the Piel pearl-bearing *Mytili* †.

The two chief facts in favour of such a view are that the sizes of the suckers and the position of the ventral sucker, in the Piel pearl-inducing Trematode, correspond more closely with those in *G. dapsilis*, while the firm brittle consistency of the body reminds one more of *G. dapsilis* than of the softer *G. bursicola*. The position of the testis in relation to the ventral sucker is also a feature of some moment. It is, of course, quite possible that two closely allied Trematodes are concerned with pearl-formation in *Mytilus*—the one derived from *Tapes*, the other from *Cardium*,—and it may be that the adults of these two species are respectively *G. bursicola* and *G. dapsilis*.

Jameson foresaw that it would be necessary to prove in some conclusive fashion the correctness of his views in regard to the life-history of the pearl-forming Trematode in *Mytilus*, and towards that end an endeavour was made in 1901 to perform feeding-experiments with a Pochard (*Fuligula ferina*), the only likely diving duck which was available at the time. These experiments were unsuccessful.

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* [I distinctly remember the occurrence of a small number of larger *Gymnophalli* in the Billiers *Œdemiæ*, though it did not occur to me at the time that more than one species might be present. I referred them all to the only species then known to me, *G. somateriæ*.—H. L. J.]

† We have not so far had an opportunity of re-examining the larvæ found in the Billiers mussels.

It was in continuance of this work that the present investigation was undertaken. Arrangements were made* to obtain some live Scoters (*Œdemia nigra*) and have them fed upon mussels infected with the pearl-inducing cercariæ. Three Scoters were purchased on our behalf by the Zoological Society from D. G. Schuijl, of Rotterdam, who stated that they were all caught on the Zwarte Water, in the province of Overijsel. In addition, four dead Scoters, stated to come from the same locality, were kindly sent to us for examination by the Superintendent of the Brighton Aquarium. With one exception, these four birds were found to be heavily infected with intestinal parasites, including a considerable number of *Gymnophalli*. These will be referred to later. Of the three live Scoters, which were housed in the Society's Gardens, one died shortly after arrival, and was found to harbour numerous specimens of *Tocotrema concavum* and immature Cestodes in its intestine. The second Scoter was removed to the Lister Institute with the object of making a feeding-experiment, but it died within 24 hours. Its intestine contained only a few immature Cestodes. The third was kept from 8th to 24th December, 1911, when the experiment was started. It was fed at intervals with mussels from the beds at Foulney, near Piel, which were infected with the pearl-inducing *Gymnophallus*. Altogether about 1000 mussels were given to it. Check-examinations of samples of these mussels showed that out of 78 mussels 32 were infected, the number of live *Gymnophalli* being 64. On February 27th, 1912, the bird was killed and thoroughly examined. The only parasitic worms present were a few immature tapeworms in the intestine and two specimens of *Metorchis xanthosomus* in the gall-bladder.

It is difficult to draw any satisfactory conclusion from the result of this experiment. At first sight the most obvious inference would appear to be that the larva in *Mytilus* does not become adult in *Œdemia*, but in some other host. This inference, however, is not without objection, for, quite apart from the evidence furnished by the close resemblance between the parasite in the mussel and in the duck, other factors may require to be taken into consideration—for instance, the somewhat unnatural mode of feeding, the unsuitability of the season, the effect of captivity, and so forth. In this connection, the complete absence of other Trematodes from the intestine is significant, as suggesting that the intestine may possibly have been cleared of parasites as a result of digestive derangements following on change of food. It is, however, useless to speculate on these matters, and for the present we are content to record the negative result of this particular experiment.

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* [Thanks to a grant from the Government Grant Committee of the Royal Society.—H. L. J.]

Our main object in submitting the present communication is to give an account of the new species of *Gymnophallus* which were encountered in the course of examining the Scoters, and also to note the occurrence of a few other Trematode parasites which have hitherto not been recorded from this host. Nicoll (1907) gives the following list of Trematode parasites from *Œdemia nigra* examined at St. Andrews:—

Gymnophallus dapsilis Nicoll.	Bursa Fabricii.
Psilostomum brevicolle Creplin.	Intestine.
Spelotrema pygmæum Levinsen.	Intestine.

The following have been recorded by other authors:—

Psilochasmus oxyurus Creplin.	Intestine.
Echinostomum revolutum Froelich.	Intestine.
Monostomum sp.	Respiratory tract.
Strigea tarda Steenstrup.	Intestine.

In the course of our examination we found the following:—

Psilostomum brevicolle Creplin.	Intestine.
Tocotrema concavum Creplin.	Intestine.
Levinseniella brachysoma Creplin.	Intestine.
Metorchis xanthosomus Creplin.	Gall-bladder.
Gymnophallus dapsilis Nicoll.	Intestine.
Gymnophallus bursicola Odhner	Bursa Fabricii.
Gymnophallus œdemiæ, sp. n.	Intestine.
Gymnophallus affinis, sp. n.	Intestine.
Gymnophallus macroporus, nom. nov.	Intestine.
Gymnophallus ovoplenus, sp. n.	Intestine.
Catatropis verrucosa Froelich.	Bursa Fabricii.

This makes a total of 15 distinct species from this single host, of which 9 are here recorded for the first time. Such a wealth of varieties of Trematode parasites in a single host is rather remarkable*.

The new species which we are describing were all obtained from the Scoters supplied by the Brighton Aquarium. The birds originally came from the same quarter as those purchased through the Zoological Society, and it is rather striking that they should have been so heavily infected, while the others were almost free from Trematodes.

GYMNOPHALLUS ŒDEMIÆ, nom. nov.

= *Lecithodendrium somateriæ* Jameson, 1902 (*ex parte*).

This was the least common of the four species, and occurred in only two of the birds examined. It is the adult form depicted by Jameson (1902, pl. xvi. fig. 11) and to which the greater part of his description (pp. 159–160) applies.

---

* [To the list must be added *Paramonostomum alveatum* Mehlis, which was obtained from a Scoter (*Œdemia nigra*) which died in the Society's Gardens on Nov. 19th, 1911.—W. N.]

The body is somewhat flat and oval, with pointed extremities. Its length is ·19–·25 mm. and its maximum breadth is usually a little more than half the length. The cuticle is entirely covered with minute spines. The oral sucker measures ·030–·037 mm. in diameter and the ventral sucker ·024–·032 mm., the ratio being approximately 6 : 5. The ventral sucker is situated at a distance of ·11–·15 mm. from the anterior end, the distance being about three-fifths of the body-length.

The pharynx is contiguous with the oral sucker and is about ·017 mm. long. It is followed by a narrow œsophagus which may be nearly twice as long as the pharynx. The intestinal diverticula diverge fairly widely and barely reach the middle of the body. The excretory vesicle consists of a very wide main stem and two long branches, which nearly reach the oral sucker.

The testes lie some distance behind the ventral sucker, but nearer it than the end of the body. They are small and irregularly globular. The vesicula seminalis is situated over the left side of the ventral sucker, usually a little in front, but sometimes extending to the posterior border of the sucker. From it issues a short pars prostatica, of the usual *Gymnophallus* type, opening on the anterior lip of the sucker.

The ovary lies on the right side, on the same level as the ventral sucker, and it is nearly as large as the sucker. The yolk-glands are situated over the anterior half of the ventral sucker. They are somewhat irregular. The uterus lies almost entirely in front of the ventral sucker, and extends forwards almost to the oral sucker, filling up most of the anterior part of the body. The eggs, which usually number 30–100, measure ·018–·020 mm. in length and ·013–·014 mm. in breadth.

GYMNOPHALLUS AFFINIS, sp. n. (Text-fig. 11.)

This species, although about the same size as *G. œdemiæ*, is at once distinguished from it by the large size of the oral sucker and by the disposition of the uterus. It measures ·2–·25 mm. in length and ·11–·13 mm. in breadth, the body being flat and oval. The ends are not usually so pointed as in the previous species.

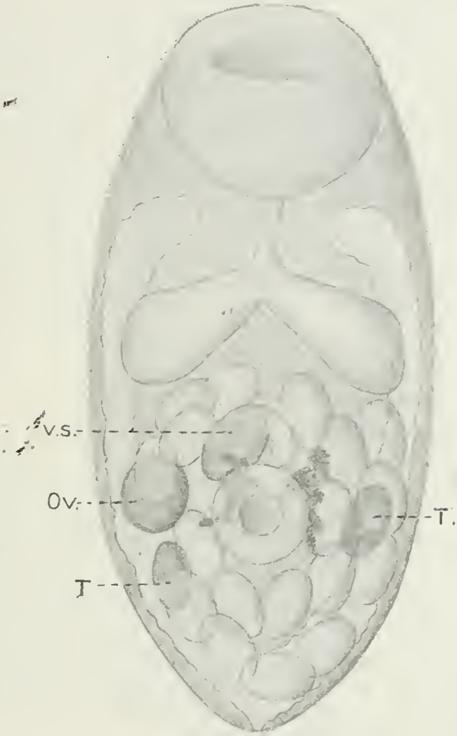
The oral sucker has a transverse diameter of ·065–·085 mm. The ventral sucker is very much smaller, measuring only ·030–·045 mm., and the ratio is usually about 2 : 1. The ventral sucker is situated ·16–·18 mm. from the anterior end, *i. e.*, a little more than two-thirds of the body-length.

The pharynx, which is contiguous with the oral sucker, measures ·015–·018 mm. in length by ·012–·017 mm. in breadth. It is followed by an œsophagus of about the same length. The intestinal diverticula diverge very widely and are usually much dilated. As a rule, they do not reach the middle of the body

The excretory vesicle is V-shaped, the main stem being very short.

The testes are two elongated oval bodies, measuring about ·025 × ·014 mm. That on the left lies on the same level as the ventral sucker, the one on the right being somewhat behind. The vesicula seminalis lies adjacent to the anterior border of the ventral sucker, and from its anterior end issues the pars prostatica.

Text-fig. 11.



Gymnophallus affinis, sp. n. Ventral view, × 400.
Ov. Ovary. *T.* Testes. *V.S.* Vesicula seminalis.

The ovary is considerably larger than the testes, measuring ·027 × ·023 mm., and is more nearly globular. It is situated almost immediately in front of the right testis and on the level of the anterior border of the ventral sucker. The yolk-glands lie over the anterior half of the ventral sucker. Each gland consists of a somewhat crescentic mass of very irregular contour, the concavities of the crescent being directed outwards.

The uterus lies mostly behind and to the left side of the ventral sucker. It seems to form a single loop, starting from the ovary, passing behind the ventral sucker and up along the left side of the body to some distance in front of the sucker, then returning in the same way. The eggs are of conspicuously large size, measuring ·021–·028 × ·013–·018 mm.

There were a considerable number of much smaller specimens (·13–·19 mm. in length) bearing a close resemblance to the foregoing. Even the smallest of them, measuring only ·135 mm. in length, was fully mature and contained about a dozen large eggs. All intermediate sizes between this and ·2 mm. were observed. It is difficult to decide whether these small specimens are the same as *Gymnophallus affinis* or whether they represent a distinct species. The differences, apart from the difference in size, are not very easy to detect. The body is altogether more plump and the various organs more packed together. The suckers are relatively larger. The intestinal diverticula extend past the middle of the body and are in contact with the ovary and the vesicula seminalis. The yolk-glands are slightly further back and appear to be partly fused or at any rate very close together, while the uterus extends forwards to the oral suckers on the left side. These differences, although noticeable, do not appear sufficiently constant to warrant the creation of a further new species, so that for the present we shall content ourselves with noting the existence of these smaller forms.

GYMNOPHALLUS MACROPORUS, sp. n. (Text-fig. 12.)

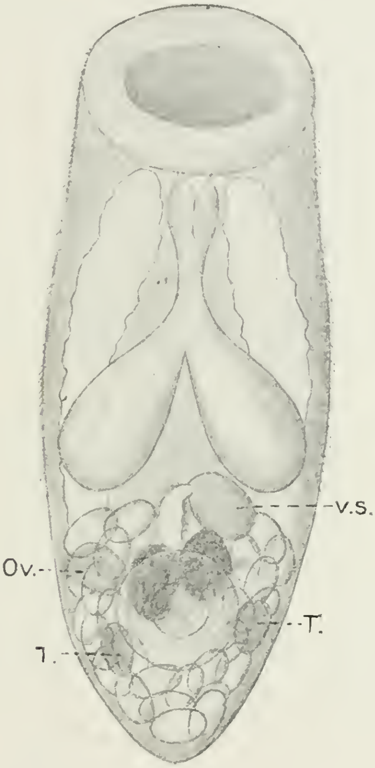
This is the largest of the four species, and measures ·4–·5 mm. in length by ·14–·23 mm. in maximum breadth. The greatest breadth is across the middle of the body, but is not very much greater than that of the oral sucker, and this gives the animal a very characteristic shape. The lips of the sucker are usually everted and project well beyond the sides of the body. From the anterior end the body tapers gradually towards the pointed tail, though there is usually a slight inflation a little in front of the ventral sucker. The oral sucker has a transverse diameter of ·14–·17 mm., the ventral sucker ·065–·075 mm. The ratio is generally about 9 : 4. The ventral sucker is situated at a distance of about ·3–·4 mm. from the anterior end of the body, *i. e.* about three-fourths of body-length.

Almost contiguous with the oral sucker is a comparatively large pharynx measuring ·040 × ·035 mm. The œsophagus is slightly longer than the pharynx, measuring ·05 mm. The intestinal diverticula are usually moderately distended and form an acute angle with each other. Their ends reach well past the middle of the body.

The excretory vesicle is **V**-shaped and the limbs are frequently enormously distended, compressing the intestine and giving the anterior part of the body a hollow appearance.

Owing to the close packing together of the eggs the ovary and testes were extremely difficult to discern. They appear, however, to have much the same disposition with regard to the ventral sucker as in *Gymnophallus affinis*, the ovary lying on the right at the level of the anterior border of the sucker, the right testis being at the posterior border and the left testis occupying an intermediate level. The yolk-glands differ from those in *G. affinis* in being much more compact, their outlines, in fact, being quite oval. They usually lie over the anterior half of the ventral sucker or a little in front of it.

Text-fig. 12.



Gymnophallus macroporus, sp. n. Ventral view, × 250.

Ov. Ovary. *T.* Testes. *V.S.* Vesicula seminalis.

The vesicula seminalis lies immediately in front of the yolk-glands, sometimes median, at other times displaced laterally. From its anterior end issues a short wide pars prostatica, running down towards the ventral sucker. The uterus is disposed around the ventral sucker and does not extend in front of the intestinal diverticula. The eggs do not show the same tendency to be massed towards the left side as is seen in *G. affinis.* They are of relatively enormous size, measuring ·029–·034 × ·015–·020 mm., the average being ·032 × ·0175 mm. There are usually about 30–100 eggs.

GYMNOPHALLUS OVOPLENUS, sp. n.

This fourth species, which is undoubtedly distinct from all the others, was characterised by its extremely minute size and by the enormous overgrowth of the uterus, which completely filled the body. The numerous eggs seen in the smallest specimen entirely obscured the other organs, rendering a description of the internal anatomy impossible. On that account, it is to some extent a matter of conjecture as to whether the species actually belongs to the genus *Gymnophallus* or not, but from its general appearance there seems little doubt that it does.

It is a very plump, subcylindrical species with somewhat pointed ends. The length is ·11–·13 mm. and the maximum breadth about ·07 mm. The oral sucker does not measure more than ·024 mm. in diameter, and it is not much larger than the ventral sucker (·019 mm.), which is situated about two-thirds of the body-length from the anterior end. The uterus fills the body, except for a small space at the tip of the tail which usually remains free. Anteriorly the eggs are packed tightly around the oral sucker, rendering it almost invisible. They measure ·016–·019 × ·011–·012 mm.

It was at first thought that these specimens were simply young forms of either *G. affinis* or *G. œdemiæ*, but it seems impossible that such can be the case. Even when the animal is only ·12 mm. long the uterus attains a stage of hypertrophied development which is never seen in either of the other species even when twice as large. It might be conjectured that either or both of these species may become prematurely ripe and that egg-production falls off later. This, however, is a hypothesis which has nothing to support it. Moreover, as has already been mentioned, specimens measuring ·13 mm. and closely resembling *G. affinis* have been found, and in them the uterus occupied only a relatively small proportion of the body.

Judging by the relative sizes of the suckers, this species is more closely allied to *G. œdemiæ* than to *G. affinis.*

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On carnaubon. By HUGH MACLEAN.

In 1910 Dunham and Jacobson isolated from ox kidney a substance which they named carnaubon. This substance was stated to be a triamino-monophosphatide in which the glycerol radicle was substituted by galactose. In my experiments on horse kidney a similar substance was obtained, which, when purified in the manner described in the foregoing paper, gave a N : P ratio of 2 : 1. It had all the properties of carnaubon and after boiling with weak mineral acid reduced Fehling's solution. Three different samples were prepared and in each case the substance proved to be a diamino-monophosphatide. It is therefore probable that carnaubon is really not a triamino- but a diamino-monophosphatide. A somewhat similar substance was isolated from egg yolk by Stern and Thierfelder. This substance did not reduce Fehling's solution after boiling with mineral acids, but as only a very small amount was isolated, and the reduction with carnaubon is not marked unless a fairly large amount is used, it is possible that this substance was the same as the diamino-monophosphatide of horse kidney. In both cases the analyses are almost identical. There is also some evidence to show that the substances isolated by Thudicum and called by him apomyelin, sphingomyelin and amidomyelin were probably of the same nature as kidney diamino-monophosphatide. This point is at present being investigated and if substantiated will be of considerable value in clearing up certain anomalies in the literature of the lipoids.

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The extraction and purification of tissue phosphatides. By HUGH MACLEAN. (*Preliminary communication.*)

The separation of the different phosphatides has always been a matter of extreme difficulty owing to their generally similar solubilities in the ordinary fat solvents, and the fact that many of these substances are intersoluble.

Formerly, the method of extraction of phosphatides consisted in treating the tissue or organ very thoroughly with alcohol and ether and precipitating the phosphatides by means of acetone. The chief substance obtained by this method was the monamino-monophosphatide lecithin. In 1907 a paper by Erlandsen appeared in which he showed that heart tissue which had been dried and extracted with ether still contained a great deal of phosphatide which could be extracted on treating the tissue with alcohol. In connection with this, Erlandsen made the very important statement that the chief phosphatide of the ether extract differed in constitution from that contained in the subsequent alcoholic extract. Since, however, the phosphatide of the alcoholic extract was found to be easily soluble in ether, the obvious explanation was that this substance existed in the tissue in the form of some combination—probably with protein. From this combination it was set free by alcohol.

In these experiments the tissue was freed from microscopic fat as much as possible, minced and spread out in thin layers on a glass plate kept at a temperature of about 30°. The drying process was accelerated by means of a current of air generated by an electric fan. The dried substance was ground to a fine powder and extracted with several changes of ether, till little or no residue remained on evaporating the ether. The finely powdered tissue was then thoroughly extracted with alcohol and a substance obtained which was isolated as a cadmium-chloride compound. The N : P ratio of the different substances obtained was as follows:

		N : P
Ether extract	(1) Lecithin	1 : 1
	(2) Cuorin	1 : 2
Alcohol extract	(3) Diamino-monophosphatide	2 : 1.

In the course of investigation of some other tissues (horse kidney) I found that the phosphatides of the ether extract were lecithin and cuorin as described by Erlandsen for heart muscle, but that the phosphatide of the alcohol extract contained very variable amounts of nitrogen and gave N : P ratios varying from 4 : 1 to 1·5 : 1. This suggested that the phosphatide present was probably contaminated by a substance or substances containing a high percentage of nitrogen, and that some light might be thrown on the nature of this phosphatide if it could be separated from this nitrogenous substance. This impurity, however, gave almost all the ordinary reactions of phosphatides, so that all my earlier attempts entirely failed to effect a complete separation though material changes in the N : P ratio were in evidence. Finally, however, it was found that repeated emulsification of the alcohol phosphatide mixture with water and precipitation by a small amount of acetone resulted in a purification of the phosphatide and on analysis it was found that the N : P ratio was the same as that of lecithin—1 : 1. The purified substance also gave all the ordinary lecithin reactions. Thus, in kidney, the phosphatide which comes out in alcohol after preliminary treatment with ether is of the same nature as the lecithin obtained in the preliminary ether extract—a monamino-monophosphatide.

A very small amount of a diamino-monophosphatide was found in horse kidney, but it differed entirely in composition and in its general physical and chemical characters from the diamino-monophosphatide described by Erlandsen in ox heart muscle. Somewhat similar results were obtained in the investigation of horse flesh ; here, as in kidney, the major part of the alcoholic extract was found to consist of a monamino-monophosphatide.

In view of the statements that have been made with regard to the curative effect of lecithin in birds suffering from polyneuritis (beri-beri), it is interesting to note that purified lecithin produces little improvement, whereas the substance with which the lecithin of the alcoholic extract is contaminated has very marked curative properties. It is probable that the explanation of the alleged curative effect of lecithin in cases of beri-beri depends on the presence of this impurity. As this substance is exceedingly difficult to separate completely, it is obvious that the majority or all of the lecithins prepared in the ordinary way must have contained more or less of it. A full account of the above will appear shortly in the *Biochemical Journal.*

THE PHOSPHATIDES OF THE KIDNEY

By HUGH MacLEAN.

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(Received June 20th, 1912)

The researches of Erlandsen[1] on the phosphatides of cardiac muscle showed that the older methods of extracting tissues with alcohol did not suffice to isolate pure substances, and that the so-called lecithin obtained was really a mixture. According to this investigator the phosphatides contained in the ethereal extract of dried cardiac muscle consisted chiefly of two substances—lecithin with a nitrogen : phosphorus ratio of 1 : 1, and cuorin with a nitrogen : phosphorus ratio of 1 : 2. Another ether soluble substance, however, was present in large amount, but was incapable of being extracted by ether until the tissue had undergone a preliminary treatment with alcohol. This behaviour towards ether suggested that this substance was present in the tissue in some combination which was split up by the alcohol, or that the ether did not penetrate the dried tissue sufficiently well to extract it. Erlandsen extracted this substance by means of alcohol after a thorough preliminary treatment of the tissue with ether, and found that the greater part of the alcoholic extract consisted of a phosphatide which differed materially in constitution from lecithin and cuorin, and contained in its molecule two atoms of nitrogen to one of phosphorus.

These observations of Erlandsen strongly supported the combination view, for if the ether were incapable of penetration as the result of purely physical conditions, it is difficult to understand why there should be any material difference in the nature of the phosphatide obtained in the ether and alcohol extracts respectively.

These results with cardiac muscle suggested that other tissues probably behave in a similar manner towards ether and alcohol, and in order to determine this the following investigation of kidney phosphatides was carried out.

Preparation of Material

Horse kidneys were used on account of their cheapness and the ease with which large supplies could be obtained. In the first experiment fifteen fresh kidneys were washed with normal saline solution and freed as much as possible from adherent fat and fibrous tissue. They were then passed through a mincing machine, and the finely divided tissue dried on a glass plate by means of a fan at a temperature of about 30°. The dried substance obtained was now dried in a desiccator over sulphuric acid and finely ground in a mill to a fine powder.

Extraction with Ether

The dried powder, which weighed 2075 grams, was shaken up with ether in a dark, well-stoppered bottle, which contained CO_2 in order to prevent oxidation. After extracting six times, the ethereal extracts were evaporated to very small bulk under reduced pressure and at room temperature. The residue, which now formed a syrupy mass, was treated with a large excess of acetone and the mixture allowed to stand for half an hour. The precipitate which formed was separated from the ether-acetone liquid, dissolved in some pure ether, and again precipitated by acetone. It was then thoroughly washed with acetone and dried in vacuo. By this treatment with acetone, most of the ordinary fat and cholesterol was separated. In separating phosphatides from fat by means of acetone, it is important to separate the precipitate from the ether-acetone solution in a short time, for though acetone first precipitates phosphatides from an ethereal solution, it gradually precipitates such substances as neutral fat and certain saturated fatty acids. The crude phosphatide obtained weighed about 29 grams.

Treatment of Crude Phosphatide of Ethereal Extract

The crude extract was dissolved in about 150 c.c. ether, and an opalescent solution obtained which on centrifuging yielded a fair amount of a white precipitate and a clear dark amber-coloured fluid. The phosphatides were precipitated from this solution by the addition of excess of acetone, and dried in vacuo. This process of dissolving in ether, centrifuging and treating with acetone was repeated four times. All the white precipitates obtained on centrifuging were mixed together, and for purposes of reference may be called 'white substance.'

The phosphatide residue was dissolved in 100 c.c. ether and gave an almost clear solution; to this 500 c.c. absolute alcohol were added, and the mixture left to stand in an ice-room overnight. Immediately on the addition of the alcohol, a precipitate formed which became more marked on standing. After sixteen hours, this precipitate was separated by filtration and washed thoroughly with alcohol. It was then dissolved in ether, precipitated by acetone and dried in vacuo.

An ether-alcohol solution (*b*) and a precipitate (*a*) were thus obtained.

TREATMENT OF ALCOHOL-INSOLUBLE SUBSTANCE (*a*)

This substance was treated with alcohol at 45° C., and the residue dried in vacuo, dissolved in ether and precipitated by acetone. After drying it was re-dissolved in freshly distilled hot ethyl acetate and allowed to stand for some hours in the ice-room, when a substance of syrupy consistency fell out. This treatment with ethyl acetate was repeated and the syrup obtained dried and analysed.

All the above operations were carried out as far as possible in an atmosphere of CO_2 to prevent oxidation; at the same time an endeavour was made to prevent any deleterious action of light by the use of dark glass desiccators and bottles.

TREATMENT OF ETHER-ALCOHOL SOLUTION (*b*)

This liquid was evaporated under reduced pressure at a temperature of about 35° C., and a small amount of a substance obtained, which was dissolved in pure ether and precipitated by acetone. This substance, which represents ordinary 'lecithin,' was dried and analysed.

TREATMENT OF 'WHITE SUBSTANCE' OBTAINED BY CENTRIFUGE

This substance was first treated with cold alcohol, when a small amount of it dissolved. On filtering, the residue was extracted with hot alcohol, when a considerable amount easily went into solution, while part seemed quite insoluble. The solution was filtered hot and the filtrate left to stand at room temperature, when a white flocculent substance separated out. This substance was filtered off, again dissolved in hot alcohol and filtered hot as before. The flocculent substance obtained was thoroughly washed with cold ether, in which it was practically insoluble; it was then treated with hot ether and dried. The part insoluble in hot alcohol was composed chiefly of inorganic salts and was not further investigated.

From the ethereal extract of kidney, three substances were now obtained.

(1) *A small amount of a substance soluble in hot alcohol but insoluble in ether. 'White substance.'*

(2) *A substance soluble in alcohol and in ether—lecithin.*

(3) *A substance insoluble in alcohol but soluble in ether—Cuorin.*

It is interesting to note that only a very small amount of ordinary lecithin was found. The greater part of the ethereal extract was composed of the alcohol insoluble portion, of which about 13 grams were isolated, while only about 2·5 grams lecithin were obtained.

(1) '*White substance*' *separated by centrifuge*

The total amount of this substance obtained was only 0·55 gram. On analysis it gave the following figures. Nitrogen and phosphorus were estimated by the methods of Kjeldahl and Neumann respectively.

Nitrogen

0·3125 gm. used 6·6 c.c. N/10 H_2SO_4 = 2·9 per cent. Nitrogen.

Phosphorus

0·1120 gm. used 7 c.c. N/2 NaOH = 3·46 per cent. Phosphorus.

N : P = 1·86 : 1.

While it is probable that this substance was not quite pure, the small quantity obtained rendered it very difficult to get quite accurate figures; it is practically a diamino-monophosphatide, and from its general appearance and properties is probably of the same nature as a similar substance described under the alcoholic extract of the kidneys.

Thus it was practically insoluble in cold ether and only very slightly soluble in cold alcohol, in hot alcohol it dissolved with ease and separated out on cooling as a flocculent substance which on drying formed a white non-hygroscopic powder. In chloroform and benzene it dissolved easily, especially on heating; cold glacial acetic acid dissolved it with some difficulty, but on heating it dissolved readily. From these solutions it was precipitated by acetone. In general it bears a close resemblance to a substance described by Stern and Thierfelder,[2] and isolated by them from egg yolk.

(2) *Alcohol and ether soluble substance—Lecithin*

This fraction had all the properties of the mono-amino-mono-phosphatide—lecithin. It was of somewhat waxy consistence, yellowish white in appearance and very hygroscopic. In vacuo it dried very quickly to constant weight, but was sticky to the touch and incapable of being powdered. In ether, alcohol, benzene, chloroform and petroleum ether it dissolved with ease and was readily precipitated from these solutions by acetone. In ether it gave a slightly opalescent solution which cleared up in a short time to a clear dark liquid.

Analyses

0·1021 gm. substance gave 0·2385 gm. CO_2 = 63·7 per cent. C.
and 0·1040 gm. H_2O = 11·3 per cent. H.

Nitrogen

0·3219 gm. used 4·5 c.c. N/10 H_2SO_4 = 2·0 per cent.
0·6123 gm. used 8·5 c.c. N/10 H_2SO_4 = 1·94 per cent.
Average = 1·97 per cent. Nitrogen.

Phosphorus

0·3202 gm. used 21·8 N/2 NaOH = 3·77 per cent.
0·2151 gm. used 16 c.c. N/2 NaOH = 3·9 per cent.
Average = 3·83 per cent.
N : P = 1·14 : 1.

It is probable that this lecithin was not quite pure, as the ratio of nitrogen to phosphorus is somewhat too high. In general, however, it agrees well with the lecithins described by Stern and Thierfelder,[2] and by Erlandsen,[1] as the following table shows.

Analyses of Different Lecithins

	Lecithin from horse kidney	Lecithin from heart muscle (Erlandsen)	Lecithin from egg yolk (Stern and Thierfelder)
C	63·7	66·29	64·63
H	11·3	10·17	10·96
N	1·97	1·87	2·08
P	3·83	3·95	3·97
N : P ratio ...	1·14 : 1	1 : 1	1·16 : 1

On hydrolysis a substance which gave the reactions of glycerophosphoric acid was obtained as well as choline, but, as in other lecithins, the yield of choline obtained was considerably less than the theoretical amount.

In order to determine the iodine value of the lecithin, a small amount was prepared specially for the purpose, as it was found that even when preserved in as complete a vacuum as possible, the iodine number gradually diminished. This specially prepared substance was isolated and purified as quickly as possible, and gave an iodine value of 85. The free fatty acids obtained from this sample by saponification with alcoholic potash in an atmosphere of hydrogen gave an iodine value of 113. It is probable, however, despite the precautions taken, that a certain amount of oxidation had occurred, so that the value obtained is perhaps somewhat low.

No saturated acids were found while bromine derivatives of acids, having three or more unsaturated bonds, were prepared.

The nature of the acids present in lecithin and other phosphatides will be discussed in a later paper.

(3) *Substance insoluble in alcohol but soluble in ether—Cuorin*

While a comparatively small amount of lecithin was found in the ethereal extract, a considerable amount of cuorin was present.

It was obtained as a yellowish transparent substance, which was exceedingly hygroscopic, but dried quickly in vacuo to a hard solid mass, which was easily reduced to a fine powder. It dissolved at room temperature in ether, petroleum-ether, carbon disulphide, and chloroform; while it was practically insoluble in cold ethyl acetate, it dissolved easily on heating; on cooling it separated out as a syrupy mass. In cold and hot alcohol it was quite insoluble. This substance oxidised with great readiness, so that it was difficult to keep it unchanged for any length of time; as it oxidised, it gradually changed its solubility, and, after a time, it became insoluble in ether and somewhat soluble in water.

It was precipitated by acetone out of its ethereal solution; platinum chloride and cadmium chloride gave insoluble combinations.

The iodine value of the freshly prepared substance was 100, while that of the fatty acids was 133. Even when kept in an evacuated desiccator the iodine value gradually diminished, so that after a fortnight it was only 83; on exposure to the air it decreased in about a week to 52.

Analyses

0·1216 gm. substance gave 0·2695 gm. CO_2 = 60·4 per cent. C.
and 0·1120 gm. H_2O = 10·2 per cent. H.

Nitrogen

0·5216 gm. used 3·8 N/10 H_2SO_4 = 1·02 per cent.
0·6210 gm. used 4·7 N/10 H_2SO_4 = 1·06 per cent.
Average = 1·04 per cent. N.

Phosphorus

0·3105 gm. used 25·2 c.c. N/2 NaOH = 4·49 per cent.
0·2108 gm. used 16·8 c.c. N/2 NaOH = 4·41 per cent.
Average = 4·45 per cent. P.
N : P = 1 : 1·93.

A comparison of this substance with the cuorin described by Erlandsen shows that it agrees exactly with the analysis of cuorin. A somewhat similar substance has been isolated by Baskoff from the liver, but though its properties are similar to those of cuorin, it gave somewhat different figures on analysis. It is probable that this substance, which Baskoff called heparphosphatid, was not isolated in a pure form, and the fact that it contains sulphur strengthens this idea.

A somewhat similar substance which I isolated from egg yolk[3] differed in its percentage of nitrogen and phosphorus from this substance, but had a N : P ratio of exactly 1:2.

COMPARISON OF ANALYSES OF DIFFERENT MONO-AMINO-DIPHOSPHATIDES HITHERTO ISOLATED

	Substance from horse kidney (MacLean)	Cuorin from heart muscle (Erlandsen)	Heparphosphatid from liver (Baskoff)	Mono-amino-diphosphatide from egg yolk (MacLean)
C	60·4	61·33	61·12	59·12
H	10·2	9·02	8·95	9·44
N	1·04	1·01	1·23	0·812
P	4·45	4·47	4·0	3·59
N : P ...	1 : 2	1 : 2	1 : 1·5	1 : 2

These figures indicate that the substance obtained from horse kidney is the same as that obtained from heart muscle by Erlandsen.

On saponification with alcoholic potash, 60 per cent. of the weight of cuorin was obtained as fatty acids, which were solid at room temperature and melted at about 45°.

They were composed of one solid saturated acid and two unsaturated liquid acids; the solid acid was found to be stearic, while the liquid acids, as in the case of lecithin, gave bromination products, indicating the presence of acids having three or more double bonds. They will be discussed, along with the acids of lecithin, in a later paper.

The other cleavage products consisted of a substance having the reactions of glycerophosphoric acid, though the amount of barium obtained on analysis of the barium salt did not quite agree with the theoretical amount. Only a very small amount of a basic substance precipitated by platinum chloride could be obtained, so it is certain that the nitrogen is not represented by choline.

From these results it is clear that the acetone insoluble phosphatides present in the ethereal extract of horse kidneys are of the same nature as those described by Erlandsen from heart muscle. In spite of the observation of Fraenkel that he could not obtain any evidence of the presence of lecithin in the kidney, there can be no doubt that a small amount is present in the ethereal extract. It will be shown later that quite a considerable amount of lecithin is present in the alcoholic extract.

The three substances present in the ethereal extract of kidney are therefore lecithin, cuorin and a diamino-monophosphatide. Cuorin was found in greatest amount, while only a small quantity of lecithin and a very small amount of the diamino-monophosphatide was isolated.

The Alcoholic Extract

Our knowledge of the phosphatides present in the alcoholic extract of the different tissues is very unsatisfactory. According to Erlandsen, by far the greater part of the extract consists of a phosphatide having a N : P ratio of 2 : 1.

This observation was based on the analyses of cadmium chloride compounds, but he was unable to isolate the substance in a free state. An attempt to liberate the phosphatide from the cadmium compound was unsuccessful, the substance obtained differing in its constitution from the theoretical composition deduced from the cadmium salt. In order to explain this result, Erlandsen assumed that the manipulations required for setting free the phosphatide resulted in a partial decomposition of this body, but it is interesting to observe that the N : P ratio of the substance obtained was 1·48 : 1.

Baskoff extracted the liver, first with alcohol, then with ether, and lastly with 96 per cent. alcohol. This second alcoholic extract was evaporated to small bulk in vacuo, and the residue dissolved in a small amount of alcohol. An insoluble fraction remained which consisted chiefly of inorganic substances. The alcoholic filtrate was now evaporated to dryness, and the residue extracted with ether, when a white substance was obtained and filtered off. This substance contained 4·16 per cent. nitrogen and 2·52 per cent. phosphorus = N : P 3·68 : 1, and was regarded by Baskoff as a jecorin-like substance. The ethereal solution was concentrated and precipitated with acetone, the precipitate dissolved in ether and some alcohol added. A precipitate was now obtained which on analysis gave 4·703 per cent. nitrogen and 1·93 per cent. phosphorus = N : P 5·07 : 1.

After filtering off this substance the ether-alcohol solution was evaporated to dryness and the residue dissolved in ether and precipitated by acetone. This fraction constituted by far the greater part of the alcohol soluble substance; it was once again purified by ether and acetone. This substance was now analysed and compared with a corresponding body obtained by Erlandsen from heart muscle.

	Baskoff's substance	Erlandsen's substance	
		I	II
P	3·39 %	3·28	3·01
N	3·91 %	3·63	3·53
P : N	1 : 2·55	1 : 2·50	1 : 2·59

These figures show that the substances contain nearly the same amount of nitrogen and phosphorus, the N : P ratio in each case being practically the same.

From this substance Erlandsen succeeded in isolating a cadmium chloride compound which gave a N : P ratio of exactly 2 : 1, while on the contrary Baskoff's cadmium compound had a N : P ratio of 1·57 : 1.

These results suggest that the substances thus obtained were not quite pure, and evidence in support of this will be brought forward later. Baskoff agrees with Erlandsen that the alcohol extract contains phosphatides with a higher nitrogen content than those of the ether extract, though he was unable fully to substantiate Erlandsen's results. It is noteworthy that both the jecorin-like substances and the alcohol soluble phosphatides all contain a high percentage of nitrogen.

Treatment of Ether Extracted Tissue

After thorough treatment with ether, the kidney substance was extracted with alcohol, first at room temperature and then at 40° C. This was repeated six times, the combined alcohol extracts evaporated off under reduced pressure and the residue extracted with pure ether. A considerable amount of substance was obtained which gave a very opalescent solution with ether. This was left to stand, the supernatant fluid treated with excess of acetone and the crude phosphatide obtained dried in vacuo.

Treatment of Crude Phosphatide Mixture

The dried crude substance, which weighed about 50 grams, was now dissolved in ether, and gave a somewhat opalescent solution which soon cleared up, leaving a small amount of a white insoluble precipitate. This was filtered off, the ethereal solution precipitated by acetone, the precipitate dried and extracted with a small amount of absolute alcohol. A very turbid liquid was obtained. On centrifuging, a white precipitate (*a*) separated, and a clear alcoholic solution was obtained.

This solution was evaporated to dryness under reduced pressure at a low temperature, and the residue taken up in alcohol. The alcoholic solution was at first somewhat turbid, but soon became quite clear. On standing in the ice-room overnight a white substance was precipitated which, on separation by the centrifuge, was washed twice with a small amount of cold alcohol = white precipitate (*b*).

This precipitate (*b*) was now mixed with precipitate (*a*), and the whole extracted twice with hot acetone. The residue was only partly soluble in ether and in alcohol.

On analysis it was found to contain 6·2 per cent. nitrogen and 1·5 per cent. phosphorus; it was not further examined.

The alcoholic solution was again evaporated to dryness, and the residue dissolved in a very small amount of alcohol by the aid of gentle heat. On standing in the ice-room overnight some more white substance separated out. After filtering, the alcoholic extract was evaporated to small bulk and about five times its volume of pure ether added, a precipitate consisting partly of a white powder and partly of a slimy mass formed and was filtered off. This precipitate was washed with ether, in which the white substance remained in suspension while the slimy portion remained at the bottom of the vessel.

The ethereal suspension was syphoned off and left to stand, when the white particles fell to the bottom of the glass. On separating the ether a perfectly white substance was obtained which, on drying, formed a white solid mass which was easily broken up to a white powder = white powder (*d*).

The slimy mass obtained was dried in vacuo = substance (*f*). The alcohol-ether filtrate was evaporated to dryness and the residue dissolved in ether, when a perfectly clear solution was obtained after standing a short time. This was precipitated by acetone, the precipitate again dissolved in ether and reprecipitated by acetone. The substance obtained gave a clear solution in alcohol, while in ether it was at first somewhat cloudy, but on standing soon became quite clear.

This substance was now dissolved in alcohol and three times its volume of acetone added. A precipitate at once formed which was filtered off, dissolved in ether, precipitated by acetone, and dried. This fraction constituted by far the greater part of the phosphatides of the alcoholic extract = phosphatide (*m*). On adding more acetone to the above acetone-alcohol solution a slight cloudiness was in evidence, but on the addition of great excess of acetone a white sticky substance separated, which differed in appearance from substance (*m*) above. This will be referred to as phosphatide (*p*).

As it was thought that these two fractions might differ in constitution, they were dried and analysed separately. The fraction (*m*), which was at first easily soluble in alcohol, became partly insoluble after some time, while precipitate (*p*) remained soluble for a much longer time.

From the alcoholic extract four fractions were now obtained.

(1)	Lecithin-like substance	(*m*)	about	16 grams.
(2)	Lecithin-like substance	(*p*)	,,	2·5 grams.
(3)	White substance ...	(*d*)	,,	3·1 grams.
(4)	Slimy mass ...	(*f*)	,,	3·5 grams.

SUBTANCE (*m*)

This was obtained as a yellowish white lecithin-like substance soluble in alcohol, ether, chloroform, benzene and petroleum-ether. It dried quickly to constant weight, was very hygroscopic, and had all the general properties of lecithin.

Analyses

Nitrogen—

1·0460 gm. used 36·2 N/10 H_2SO_4 = 4·9 per cent.
0·9627 gm. used 31·5 N/10 H_2SO_4 = 4·9 per cent.
Average = 4·9 per cent. N.

Phosphorus—

0·4172 gm. used 27·48 c.c. N/2 NaOH = 3·65 per cent.
0·4691 gm. used 30 c.c. N/2 NaOH = 3·54 per cent.
Average = 3·59 per cent. P.
N : P = 3 : 1.

From the nitrogen phosphorus ratio it seemed as if this might possibly be a pure phosphatide, and to determine this the cadmium chloride compound was prepared.

Five grams of the substance were dissolved in alcohol, and an alcoholic solution of cadmium chloride added as long as a precipitate formed. The alcohol was then filtered off, the precipitate washed thoroughly with alcohol, dried and analysed.

Analyses of cadmium chloride salt

Nitrogen—

0·7028 gm. used 11·8 c.c. N/10 H_2SO_4 = 2·35 per cent.
0·3504 gm. used 6 c.c. N/10 H_2SO_4 = 2·4 per cent.
Average = 2·3 per cent. N.

Phosphorus—

0·3264 gm. used 19·4 c.c. N/2 NaOH = 3·3 per cent.
0·2603 gm. used 15·9 c.c. N/2 NaOH = 3·4 per cent.
Average = 3·35 per cent. P.
N : P = 1·5 : 1.

From this result it was obvious that the substance (*m*) was not a chemical entity, and that the precipitation with cadmium chloride had separated off a large amount of the nitrogen. Analysis of 100 c.c. of the alcoholic filtrate obtained from the cadmium chloride precipitation showed that a very great excess of nitrogen was present. As cadmium chloride does not precipitate phosphatides quantitatively out of alcoholic solution, it is probable that part of the nitrogen was accounted for in this way. On the other hand, some nitrogenous substance free from phosphorus must have been present.

Analysis of filtrate

50 c.c filtrate used 31·5 c.c. N/10 H_2SO_4 = 0·88 per cent. N.
50 c.c. filtrate used 12·6 c.c. N/2 NaOH = 0·14 per cent. P.
N : P = 14 : 1.

These results proved that the phosphatide present could not be of the nature of the diamino-monophosphatide found by Erlandsen in heart muscle.

An endeavour was made to decompose the cadmium chloride compound in order to investigate the phosphatide set free, but the amount of substance at my disposal was too small to give a definite result.

Another sample of cadmium chloride phosphatide prepared from the substance (*m*) was washed very thoroughly with alcohol and gave the following figures:—

0·3590 gm. required 6·55 c.c. N/10 H_2SO_4 = 2·50 per cent. N.
0·3120 gm. required 18·6 c.c. N/2 NaOH = 3·3 per cent. P.
N : P = 1·6 : 1.

It would thus seem that thorough washing of the cadmium compound does not materially alter the result.

As already mentioned, part of the substance (*m*) became insoluble in alcohol after being kept for some time. This was dissolved in ether, precipitated by acetone and the nitrogen and phosphorus estimated. It contained 2·35 per cent. nitrogen and 3·46 per cent. phosphorus = N : P 1·5 : 1. On adding an alcoholic solution of cadmium chloride to an ethereal solution of this substance, a precipitate was obtained which had a N : P ratio of 1·2 : 1.

The inference drawn from these results was that the substance present was possibly a lecithin with a N : P ratio of 1 : 1, for since the treatment with cadmium chloride caused such a marked difference in the nitrogen percentage of the substance it was quite probable that an impurity was present, all of which was not removed. Further purification of the substance, however, by dissolving in ether and precipitating with acetone did not appreciably change the nitrogen percentage, and it was obvious that a pure substance could not be obtained by this method.

SUBSTANCE (*p*)

This was also a lecithin-like body, but much whiter in appearance than the fraction described above. It had the properties and solubilities of ordinary lecithin.

Analyses gave the following results:—

Nitrogen

0·6384 gm. used 25·5 c.c. N/10 H_2SO_4 = 5·4 per cent
0·7544 gm. used 28·25 c.c. N/10 H_2SO_4 = 5·2 per cent.
Average = 5·3 per cent. N.

Phosphorus

0·5717 gm. used 28·35 c.c. N/2 NaOH = 2·7 per cent.
0·4220 gm. used 22·74 c.c. N/2 NaOH = 2·9 per cent.
Average = 2·8 per cent. P.
N : P = 4·2 : 1.

This substance differed materially from substance (*m*), the percentage of nitrogen being much higher and that of phosphorus being considerably lower. This result also suggested that the substance was impure, but owing to lack of material a cadmium chloride combination could not be investigated.

Some of these experiments were repeated with other samples of phosphatides obtained by alcohol extraction in the manner described. It was found, however, that fractions corresponding to (*m*) and (*p*) contained different amounts of nitrogen from these fractions. This was taken as strong evidence that the phosphatide of the alcoholic extract contained impurities which had not hitherto been separated by any of the methods in use at present. After many attempts at purification of this lipoid, a method was at last found which solved all the difficulties and showed that the different results obtained were really due to the presence of nitrogenous impurities.

Purification of the Phosphatides of the Alcoholic Extract

In the course of this investigation it was observed that when the residue of the alcoholic extract was shaken up with water, the fluid became coloured, suggesting that part of the substance had passed into solution. Since phosphatides are ordinarily insoluble in water, it was thought that extraction with water might result in a purer substance being obtained, since obviously something more than phosphatide was present.

Part of fraction (*m*), described above, was therefore treated as follows:—Several grammes were taken and rubbed up in a mortar with a small quantity of water. When the substance was thoroughly broken up, more water was gradually added, and a dilute aqueous emulsion obtained. To this was added a small amount of pure acetone, when a white substance at once separated out and floated on the surface of the fluid. This was easily skimmed off, when the water-acetone fluid was found to be yellow in colour. The white substance separated was subjected to the same process several times in order to ensure access of the water to the fatty lipoid particles. The final product was thoroughly extracted with pure acetone, dried and analysed. The result was exceedingly satisfactory, as it showed that a great deal of nitrogen was got rid of by this method, while at the same time the purified substance had a N : P ratio of nearly 1 : 1.

Analyses of substance (*m*), which had been treated three times with water and acetone, gave results very similar to those obtained from ordinary lecithin.

Analyses of purified substance

Nitrogen—

0·3989 gm. used 5·8 c.c. N/10 H_2SO_4 = 2·1 per cent.

0·4770 gm. used 7 c.c. N/10 H_2SO_4 = 2·06 per cent.

Average = 2·08 per cent. N.

Phosphorus—

0·3800 gm. used 26·6 c.c. N/2 NaOH = 3·88 per cent

0·2158 gm. used 15 c.c. N/2 NaOH = 3·9 per cent.

Average = 3·9 per cent. P.

N : P = 1·18 : 1.

Thus a substance having originally a N : P ratio of 3 : 1 and containing 4·9 per cent. nitrogen had, after being three times treated as above described, only about 2 per cent. nitrogen and a N : P ratio of 1·18 : 1.

After being emulsified and precipitated six times it gave the same figures as ordinary lecithin.

Nitrogen

0·6516 gm. used 9·2 c.c. N/10 H_2SO_4 = 1·97 per cent. N.

Phosphorus

0·2900 gm. used 21·3 c.c. N/2 NaOH = 4·07 per cent. P.
N : P = 1·07 : 1.

This proved that the ether soluble lecithin-like substance present in the alcohol extract of the kidney is really a monamino-monophosphatide and not a diamino-monophosphatide as described by Erlandsen in heart muscle. This point is further discussed in the following paper 'On the purification of phosphatides.'

WHITE SUBSTANCE (*d*)

This white substance isolated as described formed on drying a whitish yellow non-hygroscopic powder. Before making any attempt to purify it, an analysis was made, which showed that it was probably an impure substance.

Nitrogen

0·3076 gm. used 6·6 c.c. N/10 H_2SO_4 = 3 per cent. N.

Phosphorus

0·4020 gm. used 13·45 c.c. N/2 NaOH = 1·85 per cent. P.
N : P = 3·6 : 1.

This impure substance was partly soluble in benzene, and in chloroform, from which it was precipitated by acetone; it was slightly soluble in ether, and gave Lassaigne's test for nitrogen and the ammonium molybdate test for phosphorus. After boiling with weak HCl it reduced Fehling's solution. No glycogen reaction was obtained, and Millon's protein reaction was also negative. In H_2O part of it seemed to dissolve; the aqueous solution contained abundance of chlorides but no sulphates. The substance was now shaken up with water several times until the filtrate gave no chloride reaction. On testing the aqueous filtrate it was found to contain a considerable amount of nitrogen. This treatment of the substance with water was very tedious on account of the difficulty of filtration. The addition of water to the powder gave rise to a more or less sticky opalescent mass like thick starch paste, which quickly closed up the pores of the filter paper. The use of the centrifuge evaded this difficulty to some extent, but as the washing proceeded, precipitation was very incomplete, probably due to the absence of salts, and some loss of substance was unavoidable.

Analyses of the purified substance showed that it differed materially from the crude material.

Analyses

0·1190 gm. substance gave 0·2980 gm. CO_2 = 68·3 per cent. C
and 0·1335 gm. H_2O = 12·46 per cent. H.

Nitrogen

0·3586 gm. used 7·7 c.c. N/10 H_2SO_4 = 3 per cent. N.

Phosphorus

0·2346 gm. used 14·55 c.c. N/2 NaOH = 3·44 per cent. P.
N : P = 1·93 : 1.

Thus a substance was obtained which had a nitrogen phosphorus ratio of almost exactly 2 : 1, and was probably the same substance as that described under the ether extract.

PROPERTIES

This white substance formed a tasteless and odourless powder, which was non-hygroscopic and preserved its properties after being exposed to the air for a considerable time. It was practically insoluble in cold or hot ether, but easily dissolved in ethyl alcohol, especially on heating; from concentrated alcoholic solution it separated on cooling to room temperature. It was insoluble in acetone but soluble in hot methyl alcohol, from which it crystallised out on cooling, as in the case of ethyl alcohol. In chloroform, benzene, glacial acetic acid and pyridine, it dissolved at ordinary temperature but very easily on heating. With water it formed an opalescent starch-like mixture, from which it was precipitated by the addition of acetone. On boiling for some time with weak HCl it reduced Fehling's solution, decolourised safranin, and gave all the ordinary sugar reactions. It contained no sulphur.

The alcoholic solution was neutral and gave a precipitate with cadmium chloride and with platinum chloride, also with lead acetate.

On boiling with acids it yielded a substance which formed a double salt with platinum chloride and gave the reactions of choline. The amount obtained was much less than that represented by the nitrogen present, amounting to somewhat less than 50 per cent. of the theory. No glycerine could be obtained, but owing to the relatively small amount of substance isolated and the difficulties of testing for this substance, no definite statement as to its absence can be made.

On hydrolysis, about 65 per cent. of fatty acids were obtained. The existence in the tissues of substances similar to the above was first suggested by Thudichum, who isolated from the brain three substances which he named apomyelin, sphingomyelin and amidomyelin.

Substances of this nature were afterwards described by Stern and Thierfelder, who isolated a diamino-monophosphatide from egg yolk, and by Erlandsen, who found a somewhat similar substance in heart muscle. Dunham and Jacobson also obtained a substance from ox kidney, which had a nitrogen phosphorus ratio of 3 : 1, but in other respects was very similar to certain of the above substances.

It has already been shown that the so-called diamino-monophosphatide isolated by Erlandsen was probably a mixture, as from a similar substance prepared from the kidney I succeeded in obtaining ordinary lecithin with a nitrogen to phosphorus ratio of 1 : 1. Erlandsen's substance also differed from the above in its physical properties, and especially in the fact that it was soluble in ether. The bodies described by Thudichum as sphingomyelin and amidomyelin were both in many respects similar to the substance isolated by me. In ether and cold alcohol they were only very slightly soluble and were precipitated from alcoholic solution by cadmium chloride. Amidomyelin was further described as a substance which on drying in vacuo formed a white mass which could be easily powdered, while sphingomyelin did not form a sticky mass, but was also capable of forming a powder. The properties of amidomyelin are not given. The diamino-monophosphatide described by Stern and Thierfelder agrees in analyses and properties almost exactly with my substance, the most marked difference being that it did not reduce Fehling's solution on boiling.

As the reduction with Fehling's solution is not very marked and is only obtained distinctly when a comparatively large amount of the substance is taken, it is not possible to say at present whether this reaction is due to traces of impurity or is really dependent on a carbohydrate radicle incorporated in the molecule.

The following table shows the results of analyses of these different diamino-monophosphatides.

	Substance from horse kidney (MacLean)	Substance from egg yolk (Stern and Thierfelder)	Substance from heart muscle (Erlandsen) reckoned from $CdCl_2$ compound	Substances from brain (Thudichum)			Substance from ox kidney (Dunham and Jacobson)
				Apomyelin	Sphingomyelin	Amidomyelin	
C	68·19	68·13	59·48	67·01	65·37	62·4	67·12
H	12·37	12·14	9·42	11·35	11·29	—	11·54
N	3·0	2·77	3·47	3·0	2·96	—	2·84
P	3·44	3·22	3·84	3·23	3·24	—	2·18
N : P ratio ...	1·93 : 1	1·9 : 1	2 : 1	2 : 1	2 : 1	—	2·9 : 1

CARNAUBON

This substance, which was described by Dunham and Jacobson as a triamino-monophosphatide, possesses all the properties of the substance isolated from the horse kidney. It is the most fully described and investigated of this class of substances and its composition is given as similar to lecithin, but having, instead of glycerine, another body—galactose—to which the fatty acids are attached. The acids isolated were carnaubic acid, stearic acid, and palmitic acid. Though described as a triamino-monophosphatide, the authors agree that the N : P ratio was often about 7 : 3, and consider the possibility of the substance being impure. In the light of the results of the present investigation, it seems probable that the substance was a diamino-monophosphatide, which treatment with water as above described would have purified.

If so, the question of these ether insoluble phosphatides is much simplified, for the available evidence suggests that all the substances of this nature hitherto isolated are of the nature of diamino-monophosphatides, and are probably identical in structure with pure carnaubon. In order to make certain that the substance isolated from horse kidney was really a diamino-monophosphatide, two more samples were prepared by different methods.

SAMPLE II

This sample was prepared in the same way as that described by Dunham and Jacobson, only it was finally extracted with water. Fresh kidneys were treated with hot 95 per cent. alcohol and the extract discarded. The residue was extracted first with 95 per cent., and then with 85 per cent. alcohol, and the mixed solutions filtered hot. They were then left to stand in the ice-room. The precipitate formed was collected, dried and dissolved in benzene, the solution filtered, and the benzene evaporated off by distillation under reduced pressure. The syrup obtained was thoroughly mixed with ether and left to stand, when a white precipitate separated. The ether was syphoned off and the residue washed with more ether. It was then dissolved in hot alcohol and left to stand, when a white substance separated out. This was treated with ether, dried in vacuo and extracted with several changes of water as described above. This final treatment with water is important, as otherwise a pure substance is not obtained. The resulting product gave the following figures on analysis:—

Analyses

0·1025 gm. substance gave 0·2567 gm. CO_2 = 68·29 per cent. C.
and 0·1132 gm. H_2O = 12·26 per cent. H.

Nitrogen—

0·3806 gm. used 7·8 c.c. N/10 H_2SO_4 = 2·9 per cent. N.

Phosphorus—

0·2380 gm. used 14 c.c. N/2 NaOH = 3·3 per cent. P.
N : P = 1·95 : 1.

Sample III

This substance was prepared as follows:—The crude alcoholic extract of the kidneys was evaporated to a syrup, excess of acetone added, and the precipitate rubbed up with ether, when an opalescent mixture was obtained. On centrifuging, a white precipitate separated. This white substance was thoroughly extracted with ether and centrifuged three times. The residue was dissolved in hot alcohol and filtered hot. The filtrate, however, was not quite clear, so it was again filtered and a perfectly clear solution obtained; a small quantity of a sticky substance, insoluble in alcohol and in ether, remained on the bottom of the flask.

This clear solution was allowed to stand, and the white substance which separated filtered off and dried. It was then emulsified with water, and after the addition of some acetone, left to stand for twenty-four hours, when a flocculent white precipitate was obtained. This process was repeated twice and the final product dried and analysed. It had the same composition as the two substances formerly described.

Analyses

0·1321 gm. substance gave 0·3292 gm. CO_2 = 68·0 per cent. C
0·1321 gm. substance gave 0·1475 gm. H_2O = 12·4 per cent. H.

Nitrogen—

0·1591 gm. used 3·3 c.c. N/10 H_2SO_4 = 2·9 per cent. N.

Phosphorus—

0·1300 gm. used 8 c.c. N/2 NaOH = 3·4 per cent. P.

N : P = 1·9 : 1.

Both these substances had the same properties as the one first described.

A comparison of their analyses shows that this substance was certainly a diamino-monophosphatide.

COMPARISON OF THREE SAMPLES OF DIAMINO-MONOPHOSPHATIDE

	No. 1	No. 2	No. 3	Average
C	68·3	68·29	68·0	68·19
H	12·46	12·26	12·4	12·37
N	3·0	2·9	2·9	2·93
P	3·44	3·3	3·4	3·38
N : P ratio	1·9 : 1	1·95 : 1	1·9 : 1	1·92 : 1

Since the white substance present in the ether extract agrees with this in composition and properties, it shows that this substance is identical with that described above.

Consideration of the properties of this substance suggest that part of it would be present in the ethereal extract.

GENERAL SUMMARY

The ethereal extract of the horse kidney contains three phosphatides lecithin, cuorin, and a diamino-monophosphatide. The subsequent alcoholic extract also contains a diamino-monophosphatide, which is the same as that obtained in very small amount from the ether extract. This substance is but very slightly soluble in ether, but there is a good deal of evidence to show that it is fairly soluble in an ethereal solution of lecithin. Thus, if the crude phosphatide residue of the ethereal extract of kidney is taken and extracted with ether, a very opalescent mixture is at first obtained, but as the lipoids present dissolve, the solution becomes much clearer. On centrifuging, a perfectly clear ethereal solution is obtained, but if the ether be evaporated off and the residue again extracted with ether, the solution so obtained is by no means clear, and some more white substance can be separated by the centrifuge; if, however, the solution is allowed to stand for some time before centrifuging, it may become quite clear. Again, when it seems as if all the white substance had been got rid of, it often happens that the addition of ether to the lipoid residue gives a solution which becomes clear only after some little time. This property of the diamino-monophosphatide suggests the probability that every sample of lecithin prepared from tissues containing this substance was contaminated by it, and it is difficult to see how complete separation can be achieved, though careful treatment with ether and quick centrifuging undoubtedly gets rid of all but traces. This substance is entirely different from lecithin and other lipoids in its physical properties, as it forms a white non-hygroscopic powder, and not a more or less sticky mass, as is seen in the case of lecithin and cuorin.

Owing to its greater solubility in alcohol, the majority of it is found in the alcoholic extract, but it is present only in comparatively small amount, and is not to be confounded with the substance found by Erlandsen in the alcoholic extract of heart muscle. Erlandsen's substance possessed all the properties of ordinary lecithin and was quite soluble in ether; it constituted also the chief bulk of the phosphatides present in the alcoholic extract. A corresponding substance is present in the kidney in comparatively large amount, but its analyses did not quite compare with that of heart muscle, and on subjecting it to purification by means of H_2O and acetone, it was found to be of the nature of ordinary lecithin, with a nitrogen to phosphorus rato of 1 : 1.

Thus the lipoids of the alcoholic extract of kidney are the same as the alcohol soluble ones contained in the ethereal extract; the alcohol insoluble substance cuorin, very soluble in ether, is naturally present in the ether extract. The alcohol extract contains other bodies with a high percentage of nitrogen, but these are soluble in water and may be separated in the manner described. The water soluble substances contain only a small amount of phosphorus and are not of the nature of ordinary phosphatides; their nitrogen percentage is very high. From these observations it follows that the subject of the kidney lipoids has been much simplified, and an extension of these results to other organs and tissues will probably show that many of the substances hitherto described as containing large percentages of nitrogen are really mixtures of simpler bodies with these nitrogenous water-soluble bodies mentioned above. Other tissues such as heart muscle, liver and egg yolk, are at present being investigated from this point of view.

CONCLUSIONS

(1) The acetone insoluble phosphatides of the horse kidney are lecithin, cuorin, and a diamino-monophosphatide—carnaubon.

All these are contained in the primary ether extract, while the subsequent alcoholic extract contains lecithin and carnaubon; cuorin, being insoluble in alcohol, is not present here. Thus, so far as solubility allows, the lipoids of the ether extract are the same as those present in the alcoholic extract.

(2) In the kidneys investigated, the ethereal extract contained much more cuorin than lecithin.

(3) A method is described whereby the complicated alcoholic extract can be purified from a nitrogenous substance. In all tissues investigated hitherto, the phosphatides of the alcoholic extract must have been contaminated by this substance; this explains many of the divergent results obtained.

(4) The diamino-monophosphatide isolated has all the properties of a substance found in ox kidney by Dunham and Jacobson, and called by them carnaubon. They state that this substance is a triamino-monophosphatide.

(5) It is probable that carnaubon is not a tri-, but a diamino-monophosphatide, and that the methods used for its isolation by Dunham and Jacobson were inefficient to obtain a pure substance. On extracting a substance obtained by the method employed by the above investigators with water, a diamino-monophosphatide was obtained.

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ON THE PURIFICATION OF PHOSPHATIDES

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In the preceding paper on 'The Phosphatides of the Kidney,' it has been shown that the chief part of the lipoid occurring in the alcoholic extract of horse kidney is not a diamino-monophosphatide as described by Erlandsen[1] in heart muscle, but a monamino-monophosphatide which contains nitrogenous impurities. The phosphatide was freed from these impurities by repeatedly emulsifying with water and adding some acetone when the lecithin separated out, leaving the contaminating substance in solution. In order to obtain fairly complete separation of the phosphatide from the water-acetone mixture, it is necessary to have some salt present, otherwise acetone fails to precipitate the lipoid from its aqueous emulsion; a trace of sodium chloride suffices, and it is only necessary to add a few drops of a weak solution of this substance to the water used for the emulsion in order to ensure good separation. It is important to make the emulsion with water which contains traces of sodium chloride, for the addition of salt after the emulsion is formed gives very unsatisfactory results.

The substance can be very easily emulsified by adding at first a very little water and rubbing up thoroughly in a mortar; more water is gradually added and after each addition the whole is well rubbed up. In this way a very fine emulsion approaching to a colloidal solution is formed, and the best results are obtained when great care is taken to have this emulsion as fine as possible. Both the nitrogen impurity and lecithin are precipitated by acetone, but very much less suffices to separate the lecithin than is required for the precipitation of the other substance; only the minimum quantity of acetone necessary to separate the phosphatide is added. The lecithin rises to the top of the liquid and can be easily skimmed off, while any of the impurity that may be precipitated remains suspended in the liquid and gradually falls to the bottom of the vessel. This separation of the phosphatide is by no means quantitative, and the water-acetone solution always contains a certain amount of lecithin. With care, however, the loss in this way is comparatively small.

As the method of purification had been tested only on kidneys which had been already extracted with ether, some experiments were made on other tissues. At the same time an extension of the kidney experiments was carried out. Since the phosphatide present in the ether extract was the same as that contained in the subsequent alcoholic extract, it is obvious that the primary ether extract, the secondary alcoholic extract, and a control alcoholic extract, without primary treatment of the tissues with ether, should on purification all give a substance with an approximate nitrogen to phosphorus ratio as 1 : 1. This proved to be the case.

Kidney Experiments

Some kidneys were minced and dried in the usual way Two portions of the dried substance were then extracted as follows:—

(*a*) With ether.
(*b*) With alcohol.
(*c*) With alcohol after ether (*a* extracted subsequently with alcohol).

(*a*) *Ether extract*

The ethereal extract was treated as before described in order to separate fat, cuorin and cholesterol. The final lipoid obtained was then subjected to the acetone-water purification, dried and analysed. The results show that the substance was a lecithin.

Analyses

0·4242 gm. used 6 c.c. N/10 H_2SO_4 = 1·9 per cent. Nitrogen.
0·2814 gm. used 21 c.c. N/2 NaOH = 4·1 per cent. Phosphorus.
N : P = 1 : 1.

(*b*) *Alcohol extract without ether*

The dried tissue was extracted six times, first at room temperature, then at 40° C. and the different extracts mixed together On standing some time a white precipitate formed which was filtered off. The clear extract was now evaporated to small bulk at 40° C. under reduced pressure. On adding ether to the residue a very muddy liquid was obtained, which was treated with excess of acetone in order to get rid of cholesterol and fat. The precipitate obtained from the alcohol-ether mixture was treated several times with cold acetone and finally dried in vacuo over H_2SO_4. This crude substance was dissolved in a small amount of ether and centrifuged, when a white precipitate was obtained. The precipitate was discarded and the clear supernatant fluid treated with acetone. The precipitate was again thoroughly extracted with acetone, dissolved in alcohol and left to stand in the ice-room overnight. A precipitate formed which was filtered off. This process was repeated until a concentrated alcoholic solution produced little or no precipitate when left to stand in the ice-room. The alcohol was then evaporated off and the residue extracted with ether. A fair amount of a slimy substance (K) remained behind at the bottom of the vessel. The ether solution was now treated with acetone and the precipitate dried. It was then emulsified and precipitated with acetone eight times, extracted with acetone, dried and analysed. It gave the following figures.

Analyses

0·4211 gm. used 6·2 c.c. N/10 H_2SO_4 = 2·0 per cent. Nitrogen.
0·2204 gm. used 16·5 c.c. N/2 NaOH = 4·1 per cent. Phosphorus.
N : P = 1·08 : 1.

This substance was therefore a monamino-monophosphatide and so far agreed with that obtained from the ether extract.

(c) *Extract with alcohol after ether*

The different processes utilised for the separation of the phosphatide are the same as described for the alcohol extract above. The final product obtained was treated with water and acetone eight times.

Analyses

0·6241 gm. used 9·5 c.c. N/10 H_2SO_4 = 2·1 per cent. Nitrogen.
0·3216 gm. used 24 c.c. N/2 NaOH = 3·9 per cent. Phosphorus.
N : P = 1·2 : 1.

A comparison of these three substances shows that the analytical figures are practically the same for all three preparations. The slight differences are not greater than can be accounted for by experimental error.

COMPARISON OF THREE PREPARATIONS

	Ether extract	Alcohol extract	Alcohol extract after ether	
			I	II*
Nitrogen ...	1·9	2·0	2·1	1·97
Phosphorus...	4·1	4·1	3·9	4·07
N : P ratio ...	1 : 1	1·08 : 1	1·2 : 1	1·07 : 1

* Sample described in paper on 'The phosphatides of the kidney.'

The results prove that the chief phosphatide of the kidney is lecithin or a lecithin-like substance, and that the method of purification adopted is capable of separating off the impurities associated with this phosphatide.

MUSCLE

Experiments similar to the above were carried out on horse muscle, and three substances corresponding to the fractions described above were obtained.

(*a*) Ether extract.
(*b*) Alcohol extract without ether.
(*c*) Alcohol after ether.

The treatment of the muscle tissue prior to the separation of the different fractions was almost entirely the same as that described for kidney. It is interesting to note that while much 'white substance' was present in the kidney alcoholic extract, little or none of the diaminomonophosphatide was found in muscle.

(*a*) *Ether extract.*

The phosphatide present, after being separated in the usual way and purified three times with water and acetone, gave the following figures.

Analyses

0·6212 gm. used 8·5 c.c. N/10 H_2SO_4 = 1·9 per cent. Nitrogen.
0·2164 gm. used 15·5 c.c. N/2 NaOH = 4·0 per cent. Phosphorus.
N : P = 1·05 : 1.

(*b*) *Alcohol extract without ether.*

The crude alcohol extract here contained a great amount of a slimy substance insoluble in ether, similar to a corresponding substance mentioned under kidney. The final impure phosphatide obtained was purified with acetone and water in the usual way, and samples examined at different stages in order to determine how many times it was necessary to emulsify the substance. The crude product, which contained originally nearly 4·0 per cent. of nitrogen, gave the following results:—

Substance emulsified and precipitated once.

Analyses

0·3562 gm. used 5·7 c.c. N/10 H_2SO_4 = 2·24 per cent. Nitrogen.
0·4245 gm. used 30 c.c. N/2 NaOH = 3·9 per cent. Phosphorus.
N : P = 1·3 : 1.

Thus a single treatment reduced the nitrogen percentage from

4 per cent. to 2·24 per cent., and gave a substance having a N : P ratio nearly the same as that of lecithin.

Although this sample of crude phosphatide had been prepared in the usual way, previous to the emulsification treatment it contained over 50 per cent. of its weight of a substance soluble in water. This was obtained from the water-acetone solution by evaporation = substance (K). The following results were obtained after the phosphatide had been purified four and eight times respectively.

Substance emulsified and precipitated four times.

Analyses

0·3802 gm. used 5·3 c.c. N/10 H_2SO_4 = 1·95 per cent. Nitrogen.
0·2609 gm. used 18·7 c.c. N/2 NaOH = 4·14 per cent. Phosphorus.
N : P = 1·04 : 1.

Substance emulsified and precipitated eight times.

Analyses

0·3750 gm. used 5·1 c.c. N/10 H_2SO_4 = 1·9 per cent. Nitrogen.
0·1790 gm. used 12·8 c.c. N/2 NaOH = 3·97 per cent. Phosphorus.
N : P = 1·06 : 1.

From these results it appears that the first purification by this method is sufficient to remove all but a small amount of impurities. Naturally it becomes much more difficult to get rid of these traces, but the substance obtained after four precipitations was just as pure, judging from the N : P ratio as that obtained after eight precipitations. If care is taken to make a good emulsion, it is probable that only traces of impurities remain after four successive treatments. A fine emulsion is essential in order to provide access of the water to impurities enclosed in the fatty globules.

(c) *Alcohol extract after ether.*

This was purified six times in the usual way and gave the same figures as the other samples.

Analyses

0·6428 gm. used 8 c.c. N/10 H_2SO_4 = 1·8 per cent. Nitrogen.
0·5069 gm. used 3 c.c. N/2 NaOH = 3·9 per cent. Phosphorus.
N : P = 1·02 : 1.

The chief phosphatide of muscle is therefore a lecithin, and the lipoid of the alcoholic extract is the same as the alcohol soluble one present in the ether extract—a monamino-diphosphatide. In muscle a great amount of water soluble substance is present in the alcohol extract, but this does not appear to be a lipoid, though it is precipitated by excess of acetone from both its alcohol and aqueous solutions. A comparison of the analytical figures with those obtained from the purified lecithin of kidneys suggests that the phosphatide present in both cases is the same.

On the nature of the nitrogenous impurity

As already described, on emulsifying the phosphatide obtained from the alcohol extract of muscle and precipitating with acetone, a considerable amount of material remained in solution. This substance was dark in colour, sticky to the touch, and exceedingly tenacious; on drying it formed a hard substance, but could not be powdered. It was exceedingly soluble in water, and in alcohol containing traces of water, but much less soluble in absolute alcohol. It appeared to be absolutely insoluble in ether, by which it was precipitated from solution as a dark, sticky mass. On adding some alcohol to its clear alcoholic solution, a faint white precipitate formed, causing an opalescence which soon cleared up again.

Analyses

Nitrogen—

0·4202 gm. used 18 c.c. N/10 H_2SO_4 = 6·0 per cent. Nitrogen.
0·2231 gm. used 9·8 c.c. N/10 H_2SO_4 = 6·15 per cent. Nitrogen.

Phosphorus—

0·3513 gm. used 9·1 c.c. N/2 NaOH = 1·44 per cent. Phosphorus.

Another sample gave 6 per cent. of nitrogen and only 0·5 per cent. phosphorus, so that it is probable that the latter is present as an impurity. On standing, its aqueous solution deposited small minute white round balls which were but slightly soluble in cold water, but dissolved easily on heating. The substance was practically insoluble in cold, but somewhat soluble in hot alcohol. In ether, chloroform and benzene, it was insoluble. With mercuric chloride it gave a voluminous precipitate. On recrystallising twice from water and alcohol, a pure white substance was obtained. This contained no phosphorus. Analysis gave the following figures:—

0·0934 gm. used 16·6 c.c. N/10 H_2SO_4 = 24·9 per cent. Nitrogen.

Its melting-point was indistinct, but the high nitrogen content and other reactions suggest that it is of the nature of a purin base. Further experiments on this substance are in progress. After the separation of the white substance, the residue obtained by evaporation of the mother liquor was treated as follows:—An alcohol solution was made and ether added when a precipitate was obtained; this was repeated twice. The substance was now dissolved in water and again precipitated by acetone; the residue was then extracted thoroughly with acetone and ether and dried. On analysis it was found to contain 6 per cent. nitrogen and only about 0·3 per cent. phosphorus. As it has been found that tissues contain a substance which cures beri-beri, some of this material was given to pigeons suffering from polyneuritis in order to test its effect. The result was very marked, as three pigeons exhibiting very severe symptoms were completely cured in a short time. Partially purified lecithin had a certain effect on these pigeons, but a dose of 1 gm. of such lecithin resulted only in an amelioration of the symptoms and no cure was obtained. As many observers have placed it on record that lecithin is more or less efficient in curing beri-beri, it is probable that here we have an explanation of their results. The curative substance is not lecithin, but is present in ordinary lecithin as an impurity. It can be separated, however, by the method above described, and a lecithin so purified has but little effect in curing beri-beri. The whole subject is being investigated and the results will be published at a later date.

CONCLUSIONS

The alcohol soluble phosphatide of kidney and muscle is lecithin with a N : P ratio of 1 : 1.

(1) Though substances containing different amounts of nitrogen are obtained in the different extracts, the application of the method described—emulsification and precipitation with acetone—gives a single product of the nature of an ordinary lecithin. No alcohol soluble substance having a higher percentage of nitrogen than that of lecithin has been found.

(2) From an aqueous extract of the nitrogenous impurity of lecithin a substance of basic nature crystallises out; after the separation of this substance the mother liquor is very effective in curing polyneuritis (beri-beri).

(3) This explains the anomalous results of many observers who have endeavoured to cure pigeons suffering from beri-beri by lecithin.

REFERENCE

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SOME FALLACIES IN THE ROUTINE TESTING OF URINE.

Being a Paper read in the Section of Pathology at the Annual Meeting of the British Medical Association, Liverpool, 1912.

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The subject of fallacies in urine testing is a large one, but from the point of view of general medical practice the routine testing of urine in many cases practically resolves itself into an examination of this fluid for protein and sugar. For practical purposes there is little doubt that the detection of these substances is of more material aid in diagnosis than any test for other pathological ingredients of urine. The detection of protein is comparatively simple, and in nearly every case a strict examination according to the methods suggested in the textbooks will settle the point. In the case of glycosuria, however, the matter stands on a different footing, and in this paper I propose to deal shortly with the subject of urine testing for sugar and the fallacies connected with it.

The great difficulty experienced in testing for sugar by the ordinary reagents is that of interpreting correctly the reaction obtained. The two tests in use in this country are Trommer's test and Fehling's test, and when sugar is present in abundance they are as satisfactory, if correctly applied, as any chemical test can be. It is otherwise, however, in the case of small amounts of sugar—here ambiguous reactions are obtained, the interpretation of which gives rise to much difficulty and confusion. The only help the textbooks give is summed up in the two words, "interfering substances," though how and why they interfere and what these substances exactly are is left to the imagination of the reader.

Trommer's Test.

Trommer's test has the advantage of simplicity; a solution of copper sulphate and sodium hydroxide are the only chemicals required. On the other hand, the test is somewhat difficult of application, and unless certain precautions are adopted quite erroneous results may be obtained. Any one can test this for himself by taking a urine of specific gravity of 1017 to 1018. Of this urine two portions of 5 c.cm. are taken, and to one is added first 1 c.cm. of a 10 per cent. solution of $CuSO_4$, and then half its volume of 15 per cent. sodium hydroxide; to the other the alkali is first added and the copper afterwards. In the case of the first urine, to which the copper was added before the alkali, it will be seen that a deep blue solution is formed, whereas in the other much of the cupric hydrate remains undissolved. On heating the blue solution a precipitate may be obtained, while the other mixture does not change. When this urine is tested by other means it is found to contain only the normal trace of sugar; it is therefore obvious that care must be taken in performing Trommer's test not to add the copper before the alkali. On the other hand, if excess of copper is added even after the alkali, a "sugar-free" urine will sometimes give a reaction. These results are attributed to the presence of creatinin by Schulz and by Salkowski. A solution of creatinin behaves in exactly the same way, as there is some evidence that this substance is the cause of the anomalous reaction. How it acts is not well understood. On the whole, Trommer's test is an unsatisfactory one from the clinical point of view, for it often gives rise to errors no matter how it is performed.

Fehling's Test.

Fehling's test depends, of course, on the same reactions as Trommer's test, only in this case the reagent itself contains the cupric hydroxide in solution. It is simpler than Trommer's test, and in practice a few cubic centimetres of the urine are boiled with an equal amount of the solution; if a red or yellow precipitate appears in a short time the test is positive and sugar is present. In many cases, however, it is very difficult indeed to decide whether or not a trace of sugar is present. On just heating the mixture of urine and Fehling's solution no change takes place, but after a minute or so it becomes opalescent and greenish in appearance, and if left to stand a slight precipitate may gradually settle out. In other cases nothing happens even after the urine is heated for a considerable time, but on standing a more or less well-marked precipitation takes place; again, after boiling for some time a dirty opalescent greenish milky liquid without any sign of a definite precipitate may be seen, or the solution may have merely changed to a greenish colour and all stages from a faint dirty greenish opalescence to a definite yellowish mixture exhibiting undoubted evidence of a precipitate may from time to time be observed in clinical work. The significance of these ambiguous reactions has not been sufficiently dealt with in the literature, and their explanation has never been seriously attempted. Some years ago I made some experiments on the subject, in order, if possible, to determine the factors on which these anomalous reactions depend, and the following explanation may help to make the matter more clear.

If we take a normal urine which gives no reaction with Fehling's solution when treated in the ordinary way, and add to this urine a small amount of sugar, it is often found that no evidence of the added sugar is indicated by this test. Since Fehling's solution is capable of detecting one part of glucose in 125,000 parts H_2O (0.008 per cent.), it is obvious that lack of delicacy is not the cause of the failure in this case. Since, therefore, small amounts of sugar capable of giving a distinct reaction in aqueous solution give no reaction when added to an equal amount of urine, it is obvious that urine must contain something which prevents small quantities of sugar from reacting normally with Fehling's solution. This substance is creatinin, which is normally present in urine in sufficient abundance to interfere with Fehling's test. Creatinin is capable of holding in solution the reduced suboxide of copper formed as the result of the action of sugar on the cupric salt, and so the mixture gives no evidence of a precipitate indicating the presence of sugar.

For this reason normal urine, which contains an appreciable amount of sugar, gives no reaction with Fehling's test. It will be seen that the action of creatinin is in this case more beneficial than otherwise, for it prevents mistakes being made with physiological urines containing only the normal amount of sugar.

The action of creatinin, however, does not end here; it further possesses the property of materially modifying the nature of the precipitate when there is a slight excess of sugar present. If, for instance, we take a 0.3 per cent. sugar solution, and to one portion of this add a few milligrams of creatinin, it will be found that the precipitate obtained on heating with Fehling's solution is distinctly yellow and finely divided; without the addition of creatinin a dense red granular precipitate separates out.

The explanation generally put forward is that the yellow precipitate consists of reduced cuprous hydrate, while the red one is composed of the reduced oxide. Cuprous hydrate, however, is so unstable that it is difficult to accept this explanation; again, it entirely fails to explain all the different shades between green and yellow so often observed. It is more probable that the colour of the precipitate formed depends on the fineness of the granules, for microscopic examination of these different coloured precipitates shows that some relationship exists between the colour of the precipitate and the relative size of the particles. In a urine which after boiling for some time gives a dirty greenish, opalescent solution, the modified colour is due to the fact that the precipitate of cuprous oxide is present in an exceedingly finely divided state. In the case of a greenish-yellow precipitate the particles are still very fine but rather coarser than in the last; with a yellow precipitate they are somewhat larger, and this increase in the size of the particles goes on until in the case of a distinct red precipitate the size of the individual granules is much more marked. It is probable that all the precipitates are really composed of cuprous oxide. The causation of these ambiguous reactions is easily explained in the light of the above observations. In examining a urine containing just the normal trace of sugar the equivalent amount of Fehling's solution is reduced in the ordinary way, but the creatinin present holds the reduced cuprous oxide in solution, so that no change is apparent to the eye. When the urine contains just a slight excess of sugar the creatinin present is not sufficient to hold the cuprous oxide in solution, but it modifies the nature of the precipitate, so that it separates out in an exceedingly finely divided form. These fine particles floating in the liquid give a dirty milky greenish appearance to the fluid. These ambiguous precipitates are just modifications of the normal form and nearly always indicate a slight excess of sugar above the amount present in normal urine. In concentrated urines these ambiguous reactions constantly occur without in any way indicating that the total amount of sugar excreted is at all in excess of the normal, though the amount per cubic centimetre of urine is naturally somewhat above the average.

Of course, it is obvious that the presence of a sufficient amount of some other reducing body—glycuronic acid—would act in the same way, but observation shows that sugar is really the substance which most often gives these modified reactions.

Again, in certain urines it is only after boiling with Fehling's solution for some time that a result is obtained; this is due to the fact that though the creatinin holds in solution the cuprous oxide formed during the initial stages of boiling, yet the creatinin itself is gradually destroyed by the alkali, and a point is reached at which the creatinin present can hold no more cuprous oxide in solution; at this point a precipitate settles out.

Even normal urine, when boiled long enough with Fehling's solution, will give a distinct precipitate, so that Fehling's test is positive in every urine if the heating is sufficiently prolonged.

The following extract from one of my papers on the subject indicates shortly what happens in normal urine, and exactly the same mechanism is present in pathological urines with excess of sugar:

> The chief reducing substances present in all normal urines are uric acid, carbohydrate material (of which the larger part seems to be dextrose), and creatinin. Uric acid and sugar, however, differ greatly from creatinin in regard to the time required to cause reduction. Both substances, in the percentage in which they occur in normal urine, are capable of effecting reduction almost immediately on the boiling point being reached; in the process of reduction they undergo destruction.
>
> Creatinin, on the other hand, reduces very slowly indeed. When a normal urine is boiled with Fehling's solution, the uric acid and sugar present almost immediately reduce their equivalent amounts of the solution; no effect is perceived owing to the fact that the reduced suboxide is held in solution

by the creatinin; after boiling for a very short time the full reducing effect of both acid and sugar is completed. On continued boiling the creatinin gradually causes further reduction, at the same time becoming gradually diminished in amount; part of it is probably converted into creatin while part is destroyed.

Ultimately a point is reached at which the suboxide reduced by the sugar and uric acid, added to that reduced by the creatinin itself, is no longer capable of being held in solution by the amount of creatinin and its derivatives actually present in the urine at that moment; at this point precipitation occurs.

Thus it will be seen that the reaction obtained from a normal urine is very similar in character to that obtained from a urine containing more than the normal amount of sugar, the chief difference being that the more sugar present the quicker the reaction occurs.

In the presence of great excess of sugar the effect of creatinin is of course quite obscured and of no practical importance. It will be seen that the influence of creatinin on Fehling's solution is very marked and important, though somewhat different from that ascribed to it in the textbooks, where its action is generally considered in relation to its direct reducing power. This direct action occurs but slowly, and is therefore very insignificant when Fehling's test is used in the ordinary way; its direct inhibitory action, however, explains many points which are otherwise obscure, and for which no definite explanation has been forthcoming.

In general, therefore, it may be taken for granted that a urine which gives an ambiguous reaction with Fehling's solution contains a slight excess of sugar per c.cm. This may be due to concentration or other causes, and its significance must be estimated from a consideration of other details of the case. When certain drugs are being given similar reactions may, of course, occur.

It is hoped that these remarks will make clear the mechanism of anomalous reactions obtained in testing urine for sugar with Fehling's solution, and so render their interpretation more easy. In doubtful cases fermentation with yeast, formation of the osazones, and other more or less complicated tests must be applied.

On some Causes of Glycosuria.

With regard to the significance of a slight excess of sugar in the urine, it may be unimportant or of great importance. The facility with which certain factors produce glycosuria has hardly been sufficiently realized, and in this direction alcohol has a very pronounced effect. I once examined a number of urines from patients suffering from the effects of alcohol—most of them acute cases—and in almost every instance sugar was present. This led to further experiment, and it was found that in many cases the ingestion of comparatively small amounts of alcohol induced fairly well marked glycosuria; in some people really trifling amounts produce this effect. In the case of six temperate persons who had dined out, and whose urines I had an opportunity of examining, sugar in small amount was found in five of these. All the cases cleared up in a day or two.

Again, after certain drugs, such as $CHCl_3$, it is known that the urine reduces Fehling's solution, and this has been ascribed to the presence of glycuronic acid, but in many cases at least it is due to sugar, for nearly all these urines are fermentable.

These urines show only occasional sugar reactions, and the presence of sugar seems to be of little importance; but when a urine constantly gives even an ambiguous Fehling's reaction, and no cause such as drugs, alcohol, etc., can be found, it may be of grave significance as indicating the onset of diabetes.

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THE UTILISATION OF DIFFERENT SUGARS BY THE NORMAL HEART. By HUGH MACLEAN AND IDA SMEDLEY.

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Although many indirect observations supported the assumption that contracting muscle utilises sugar, it is only within recent years that direct proof of the consumption of sugar by muscle has been furnished. The experimental work carried out on voluntary muscle yielded on the whole unsatisfactory results, but an investigation into the action of the heart on sugars furnished conclusive proof that contracting cardiac muscle was capable of consuming glucose. In 1907, Locke and Rosenheim(1) perfused an isolated rabbit's heart with saline solution to which some glucose had been added and by quantitative determination of the amount of sugar present before and after the experiment furnished a satisfactory demonstration of the utilisation of sugar by the heart muscle. Confirmation of these results has been furnished by such observers as Rohde(2) and Gayda(3) and in a recent interesting publication, Knowlton and Starling(4) showed that the heart of the dog when fed with normal blood *in situ* was capable of consuming a considerable quantity of glucose.

In the light of these experiments there remains no further doubt that the contracting cardiac muscle is capable of utilising glucose, and all controversy with regard to this point may be taken as finally closed. Since however glucose is probably the only sugar which is normally present in blood it was of interest to determine whether this action was entirely specific and limited to glucose, or whether the other sugars which are capable of utilisation as foods in the body are also capable of being assimilated by the heart.

The animals used were rabbits and dogs. In each case the heart was isolated and perfused with saline solution containing sugar in an apparatus similar to that used by Locke and Rosenheim. Locke's modification of Ringer's fluid was used for perfusion.

In experiments of this nature it is exceedingly important to guard against the introduction of bacteria, otherwise the result may be entirely vitiated. As a general rule, when the perfusion was limited to short periods no difficulty was experienced, but in many cases after five to six hours bacteria were found in considerable numbers. These results were excluded and the majority of our later experiments limited to four hours or less. Before each experiment the whole of the apparatus was sterilised in the autoclave. In several recorded cases it appears that the consumption of sugar per hour increased with the duration of the experiment. Such effects are undoubtedly the result of bacterial action, for similar results were observed in certain of our experiments but in every case where this occurred, bacteria capable of fermenting sugar were present.

Heart of rabbit. The heart was quickly removed from the pithed rabbit and washed out with normal saline solution in order to remove excess of blood. A cannula was then tied into the aorta and about 100 c.c. of the same sugar-containing solution as that used in the actual experiment was passed through, the solution having been previously oxygenated. The apparatus was washed out with a similar solution which was circulated through it for a few minutes. The heart was then put into the chamber and perfused in the manner described by Locke and Rosenheim. A sample was taken after ten to fifteen minutes and the sugar estimated. At the close of the experiment the percentage of sugar remaining in the fluid gave an indication of the amount used by the heart.

The following sugars were investigated.

Disaccharide.		Maltose.
Hexoses.	(Aldoses)	Glucose, Mannose, Galactose.
	(Ketose)	Lævulose.
Pentose.		Xylose.
Triose.		Dihydroxyacetone.

The results obtained are shown in Table I.

The sugar was estimated by Bertrand's method and the different sugars calculated as glucose. The weight of each heart was from six to ten grms.

In four hours the quantities of glucose removed varied from 16 to 29 mgrms. In three hours 14 mgrms. of mannose disappeared so that its rate of removal is somewhat similar to that of glucose. The sample of mannose was prepared in the laboratory from ivory nut. Galactose

TABLE I. *Heart of Rabbit.*

Sugar	Amount of fluid circulated after 1st sample withdrawn (c.c.)	% sugar in solution	No. of c.c. $KMnO_4$ solution required by 20 c.c. sugar solution after perfusion for				No. of milligrams of sugar removed in		Remarks Weights of hearts = 6 to 10 grms.
			15 m.	2 h. 15 m.	3 h. 15 m.	4 h. 15 m.	(a) 3 hrs.	(b) 4 hrs.	
Glucose	100	·15	5·3	—	—	4·6	—	·0185	—
	100	·2	7·7	—	—	7·1	—	·016	—
	160	·2	8·3 8·3	—	—	7·6 7·7	—	·029	—
	140	·2	7·5 7·7	—	—	6·9 6·95	—	·026	—
Mannose	100	·1	6·3	—	5·8	—	·014	—	From methyl-phenyl-hydrazone.
Galactose	100	·1	3·9	—	—	3·65	—	·0065	Purified by yeast fermentation.
	—	·1	4·65 4·65	—	—	4·40 4·45	—	·006	,, ,, ,, ,,
	—	·1	3·55	—	—	3·35	—	·0045	Purified by methyl-phenyl-hydrazone.
	—	·1	4·0	—	—	3·7 3·75	—	·0075	—
	—	·15	5·65 5·55	—	—	5·45	—	·004	—
	—	·1	3·35	—	—	3·25	—	·0025	—
	—	·2	7·5	—	—	7·3	—	·0055	—
	—	·2	6·7	—	—	6·6 6·55	—	·0035	—
Lævulose	100	·2	7·05 7·05	—	—	6·95	—	·002	—
	—	·15	5·3	—	—	5·3	—	·000	Pure from inulin.
	—	·15	5·55	—	5·50	—	·001	—	,, ,, ,,
	—	·2	6·8	—	—	6·8	—	·000	,, ,, ,,
Di-oxy-acetone	100	·2	7·15 7·15	—	—	7·25	—	+ ·0025	—
	—	·15	5·4	—	—	5·5 5·5	—	+ ·002	—
	—	·15	5·6 5·7	—	—	5·6 5·7	—	·000	—
Xylose	100	—	6·6	—	—	6·6	—	·000	—
Maltose	100	—	3·0	—	—	2·95	—	·0005	—
	100	—	3·6 3·6	—	—	3·55	—	·001	—

is apparently utilised to a much less extent—from three to eight mgrms. in four hours. In this case the quantities which disappeared were so small that at first we were inclined to ascribe them to traces of glucose present in the sample. Two methods of purification were therefore used, the material worked with being Kahlbaum's pure galactose. The galactose was converted into its methyl-phenyl-hydrazone and the sugar again liberated by boiling with benzaldehyde according to Fischer's directions. In the second method the galactose was fermented by yeast and recrystallised from alcohol several times. In both cases the rate of disappearance was similar. From these results it would appear that the cardiac muscle is capable of utilising pure galactose though to a much less extent than glucose or mannose.

The sample of lævulose was Kahlbaum's "pure from inulin." Here no disappearance was observed even after four hours, so that apparently the rabbit's heart is incapable of dealing with lævulose. In view of certain results obtained with the dog's heart and described later, it is possible that a very slight effect may be produced but this is so small as to be within the region of experimental error. If any disappearance does take place it is so exceedingly minute as to be incapable of estimation by the method used. It was thought of interest to try another ketone besides lævulose, and di-oxy-acetone was investigated. Here again there was no disappearance, so it would appear that the rabbit's heart is incapable of dealing with keto-sugars. In the case of xylose and maltose no disappearance took place.

In conducting experiments with different sugars it is of very great importance to have pure samples. Thus commercial galactose may contain glucose and the same holds good with regard to lævulose. In the case of the latter the difficulty is avoided by using lævulose prepared from inulin. Some of our earlier experiments yielded anomalous results which we ascribed to the presence of impurities. When pure samples were taken, as in the experiments described above consistent results were generally obtained. Unless the purity of the sugar used is assured no importance can be attached to the quantitative determination of the amount consumed.

Heart of dog. Owing to the small size of the rabbit's heart, it was thought advisable to use the heart of a larger animal and thus magnify the amount of sugar which disappeared. For this purpose we used the dog's heart. The method of procedure was exactly the same as that described above for the heart of the rabbit. The dog was killed and the organ isolated as quickly as possible; it was then washed and perfused as before.

TABLE II.

Heart of dog.

	Weight of heart in grms.	Sugar used	Amt. of fluid circulated after withdrawal of 1st sample (c.c.)	% sugar (approx.)	No. of c.c. $KMnO_4$ required by 20 c.c. sugar solution (Bertrand's method) after circulation for				No. of mgrms. of sugar removed during			Remarks
					15 m.	1 h. 15 m.	2 h. 15 m.	3 h. 15 m.	1 hr.	2 hrs.	3 hrs.	
(1)	80	Dextrose	200	·2	7·35	6·25	5·40	4·40	60	100·5	142·9	—
(2)	70	,,	200	·2	7·65 7·70	—	—	3·7 3·6	—	—	312	—
(3)	11	,,	125	·3	11·0	—	—	9·8	—	—	38·1	Pup 7 weeks old.
(4)	58	Mannose	225	·2	7·35 7·30	6·65	6·05	5·20	41	73	114	—
(5)	88	Galactose	275	·2	7·3	6·7	6·3 6·4	—	36	55	—	—
(6)	11	,,	125	·2	7·0	—	—	6·65	—	—	13	Pup 7 weeks old.

TABLE III.

Action of Dog's Heart on Laevulose.

	Weight of heart in grms.	Sugar used	Amount of fluid circulated after withdrawal of 1st sample (c.c.)	% sugar (approx.)	No. of c.c. $KMnO_4$ required by 20 c.c. sugar solution (Bertrand's method of estimation) after circulation for				No. of mgrms. of sugar removed during			Remarks
					15 m.	1 h. 15 m.	2 h. 15 m.	3 h. 15 m.	1 hr.	2 hrs.	3 hrs.	
(1)	80	Lævulose	200	·2	7·1 7·1	—	—	5·5 5·5	—	—	87	—
(2)	70	,,	200	·2	7·1 7·3	—	6·6 6·8	—	—	27	—	—
(3)	72	,,	200	·2	7·4 7·3	6·3	5·5	4·6	61	100	138	—
(4)	110	,,	200	·2	5·9 5·95	5·6	5·45	—	16	23	—	—
(5)	137	,,	220	·2	6·95	6·0	5·25 5·35	—	56	93	—	—
(6)	100	,,	250	·1	3·6	—	—	2·25	—	69	—	Solutions were cleared with dialysed iron before estimating the sugar.
Control with frozen heart.												
(7)	64	Lævulose	250	·2	6·5	—	6·45	—	—	2	—	

With glucose, mannose and galactose results corresponding in general with those described under the rabbit's heart were obtained, the total quantities disappearing being of course greater in proportion to the greater size of the heart.

The results are shown in Table II.

Lævulose, which was unacted upon by the rabbit's heart, invariably disappeared in the heart of the dog, but the amount varied considerably. This is seen in Table III. It was thought possible that this might be due to a mechanical retention of lævulose by the heart. *A priori* we might expect some slight retention since lævulose is not normally present in the heart muscle and a certain amount might therefore be retained in accordance with the ordinary laws of diffusion. In order to test this the following experiment was devised.

A dog's heart immediately after its removal from the body was washed free from blood, placed in ice, and allowed to remain there during an hour. It was then placed in the apparatus and perfused with a 0·2 °/₀ solution of lævulose in the usual manner. After two hours only two mgrms. of lævulose had disappeared. The amounts removed by normal hearts in the corresponding period were from 23 to 100 mgrms. We conclude therefore that the disappearance observed is not caused by a mechanical retention of the sugar.

GENERAL REMARKS.

From the above results it appears that the utilisation of sugar by the heart is not confined to glucose: other sugars are also consumed but apparently not to the same extent except in the case of mannose. Again in the dog and rabbit the results are not quite parallel. Lævulose, which is apparently unattacked by the heart of the rabbit, is removed by the dog's heart in some cases almost as well as glucose. After we had completed the above investigation, a paper by Neukirch and Rona(5) appeared dealing with the utilisation of sugar by the heart of the rabbit and cat. Their results with rabbits are in general similar to those we have obtained: lævulose and maltose were not attacked nor were lactose and cane sugar which they also investigated. In their research with galactose, this sugar seems to have been used up to a greater extent than was the case in our experiments: otherwise our results are in accordance. In the case of the cat's heart two experiments are described lasting respectively for nine and six hours; in both cases during the first three hours very little disappearance of sugar was observed whilst in the subsequent periods considerable disappearance in the amounts of sugar took place. From this it is concluded that a reserve substance present in the cat's heart is first consumed and that subsequently sugar is destroyed. It seems to us however that no definite conclusion can be drawn unless bacterial infection has been rigidly excluded, for the difficulty experienced in obtaining sterile conditions after several hours is very great (cp. Harden and MacLean(6)). In our heart experiments bacteria were often in evidence after five to six hours although the ordinary precautions to ensure asepsis were observed.

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THE BEHAVIOUR OF THE DIABETIC HEART TOWARDS SUGAR. By HUGH MACLEAN AND IDA SMEDLEY.

(*From the Institute of Physiology, University College, and the Lister Institute, London.*)

SINCE Minkowski in his classical experiments showed that the normal power of the organism to utilise sugar is dependent on the presence of the pancreas, it was of interest to determine what effect the heart of the depancreatised and diabetic animal would have on sugar. An answer to this question was furnished by Knowlton and Starling[1], who showed in a recent publication that the heart of the depancreatised dog very quickly lost the power to consume sugar. As early as the second day after the extirpation no appreciable consumption of sugar could be observed. Their experiments were conducted under conditions in which the quantitative determination of small amounts of sugar was very difficult, since blood was used for perfusion and the difficulty of accurately estimating sugar in blood is well known. As we were perfusing hearts at the same time with saline solution the accurate estimation of sugar was comparatively simple, and, at Prof. Starling's suggestion, we carried out some experiments on the hearts of diabetic dogs. The operations were performed by Prof. Starling and the subsequent manipulations were similar to those described in the preceding paper.

The results obtained are shown in the following table. The first experiment refers to the normal heart and furnishes a record for comparison. In two cases some sugar was used though the amount was very considerably less than that consumed by the normal heart in the same time. In the other four experiments there was practically no evidence of any disappearance, the slight change obtained being well within the region of experimental error. As the result of these six observations it would seem that in many cases the diabetic heart entirely loses the power to utilise sugar, but in certain cases, while this power is considerably reduced, it is not entirely lost. In the latter it is possible that a longer period of time between the operation and the perfusion of the heart would result in complete inability to remove sugar. A noteworthy point in these experiments was the extremely good condition of the heart; in every case, with the exception of No. 7, the heart beat very strongly and regularly.

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[1] This *Journal*, XLV. p. 146. 1912.

	Weight of heart in grms.	Amount of fluid circulated in c.c. after withdrawal of first sample	Percentage dextrose	No. of c.c. $KMnO_4$ required for 20 c.c. sugar solution (estimated by Bertrand's method) after circulating for				Total amount of sugar removed in mgrms. during			Remarks
				15 m.	1 h. 15 m.	2 h. 15 m.	3 h. 15 m.	(*a*) 1 h.	(*b*) 2 h.	(*c*) 3 h.	
1.	80	200	0·2	7·35	6·25	5·40	4·40	60	106	159	Normal heart.
2.	50	100	0·15	5·8 5·85	—	—	5·0 5·0 5·05	—	—	21	2nd day after operation.
3.	54	130	0·1	4·15	—	—	3·75	—	—	13	3rd day after operation.
4.	40	225	0·3	8·65 8·8	8·8	8·55 8·5	8·6 8·4	0	1	1	2nd day after operation. 15 c.c. sugar solution used in estimations.
5.	75	225	0·3	10·5	10·45	—	—	3	—	—	3rd day after operation.
6.	47	250	0·3	11·25 11·0	11·1	—	—	0	—	—	2nd day after operation.
7.	61	250	0·3	10·7 10·6	10·6 10·6	—	—	3	—	—	3rd day after operation. Pregnant.

Effect of pancreatic extract on the diabetic heart. In the experiments of Knowlton and Starling it was shown that the addition of pancreatic extract to the diabetic heart restored the power to consume sugar. Our results have not been entirely conclusive and many more experiments in this direction are wanted. In two cases in which 10 c.c. of extract of dog's pancreas prepared according to Starling's directions were added to the perfusion fluid the power to utilise glucose was to a certain extent restored. In certain cases, cat's pancreas was used and here we failed to observe any effect. With such a limited number of experiments no definite conclusion with regard to the action of cat's pancreas can be given, as it is not improbable that certain conditions, of which we are at present ignorant, must be present in order to obtain an active pancreatic extract. The general indications however are that the power to consume sugar is to some extent restored by the addition of pancreatic extract, but until we know something more of the factors governing the formation and extraction of the active pancreatic substance it is difficult to arrive at a definite conclusion. The following are our results with pancreatic extract.

Exp. 1. Dog was depancreatised three days before experiment. Heart removed and power to consume glucose tested. After one hour 3 mgrms. were consumed; 10 c.c. of pancreatic extract (from dog) were then added, the total volume of fluid being 275 c.c. 50 c.c. withdrawn as sample after 15 minutes.

After 15 mins. 20 c.c. solution contained 53·8 mgrms. glucose
After 1 hr. 30 mins. more, 20 c.c. contained 49·5 ,, ,,
4·3 ,, ,,

i.e. total consumption of sugar 4·3 × 11·25 = 48·37 mgrms. in 1·5 hours. Weight of heart = 75 grms.

Exp. 2. Heart 2nd day after extirpation of pancreas. In one hour used no sugar. 30 c.c. extract of cat's pancreas used; tested for 1 hour 20 mins.

15 mins. after addition of pancreas extract 20 c.c. contained 54·5 mgrms. glucose.
1 hour 20 mins. afterwards 20 c.c. contained 54·2 ,, ,,

In this case no effect was produced. Weight of heart = 47 grms.

Exp. 3. Heart 3rd day after operation. During 1st hour's perfusion no sugar used. Then cat's pancreatic extract (20 c.c.) added. Heart was beating badly and after addition of pancreatic extract soon stopped. No sugar was consumed. Weight of heart = 61 grms.

In the following two experiments pancreatic extract was added immediately without testing whether the heart still retained any power to use sugar.

Exp. 5. Heart used 2nd day after operation. 300 c.c. containing total extract of dog's pancreas perfused. After 15 mins. 60 c.c. withdrawn.

After 15 mins. 20 c.c. contained 54·95 mgrms. glucose.
After 2 hours 20 c.c. contained 50·95 ,, ,,

i.e. total consumption in 2 hours = 4 × 12 = 48 mgrms.

Exp. 6. Heart 3rd day after operation. Here cat's pancreas was used but after one hour no consumption of sugar was observed.

CONCLUSIONS.

(1) The normal power to consume sugar is absent or largely diminished in the heart of the depancreatised dog.

(2) There is evidence that this power can sometimes be restored by the addition of pancreatic extract. These results in general confirm those of Knowlton and Starling.

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No 38

A Note on the Protozoa from Sick Soils, with some Account of the Life-Cycle of a Flagellate Monad.

By C. H. MARTIN, M.A., late Demonstrator in Zoology at Glasgow University and at the Imperial College of Science.

(Communicated by Prof. E. A. Minchin, F.R.S. Received May 10,—Read June 20, 1912.)

[PLATE 10.]

Of recent years a great deal of attention has been attracted to the subject of sick soils, *i.e.* soils of which the produce has fallen off without the decrease being definitely attributable to the lack of plant-food in the soil. In this preliminary note I should like to deal shortly with some aspects of this question from a zoological point of view, and also to describe the life-cycle of a flagellate monad which happened to be one of the organisms from this source, of which I have succeeded in obtaining pure animal cultures. I hope to describe cultures of this and other forms in greater detail in a forthcoming paper.

The literature on this subject from a botanical and chemical standpoint will be found in a paper by Dr. Russel and Dr. Hutchison "On the Effect of Partial Sterilisation of Soil on the Production of Plant-Food,"* and it is particularly to this work that we owe the insistence on a hypothesis which is full of interest to the zoologist, namely, that the activity of protozoa in these soils may in many cases be a factor in bringing about soil-sickness.

It is obvious that one of the first steps in the discussion of this problem must be the elucidation of the normal protozoan population of a soil.

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* 'Journal of Agricultural Science,' 1909, vol. 3, Part II.

It must, I think, be clear that on the surface of ordinary soils practically any form of protozoan may be found either in the encysted or in the active condition. If small surface pools are formed on well-manured soil it is obvious that a large number of protozoa, including the larger ciliates, may thrive. The question of the ciliates has already been dealt with by Mr. Goodey,* who was, as far as I am aware, the first to describe some of the protozoa of sick soil. But it seemed to me that, in spite of his paper, much remained to be done, particularly in connection with the smaller amœbæ and the flagellates, which must, I believe, play the most important *rôle* in the causation of this phenomenon. It seems probable *à priori* that the most common limiting factor as regards the activity of protozoa in the soil is the average quantity of water. It is generally assumed that in a fairly dry soil—that is, a soil that feels quite dry to the touch—there is 20 per cent. of water by volume; but as this water is largely present in the soil as a surface-film on the small soil particles, it is evident that the protozoa capable of leading a trophic life under these conditions must be either of a very small size or else must have developed some particular protective mechanism against desiccation. It seems to me that there are two possibilities for a protozoon that is going to do any marked harm in a soil during a year (on the assumption that Russel and Hutchison's hypothesis that soil-sickness is due to the ingestion of soil-bacteria by protozoa)—either the protozoon must be satisfied with a very low percentage of water in the soil during its trophic life, or else it must have a capacity of readily encysting, together with the capacity of reproducing with enormous rapidity as soon as the soil becomes saturated with the necessary amount of moisture. It is quite possible that all the harmful protozoa will be found to possess both the above faculties to a certain degree—probably coupled, if they are to make the best use of the water of the surface-film, with a small size. In the case, however, of the soils of sewage farms, which are frequently saturated with water, any of the protozoa, even the larger ciliates, would be capable of acting with a certain amount of efficiency as a check upon bacterial life. In order to limit the problem, I decided to examine a soil which, though probably never very dry, was yet never in the waterlogged condition of the soil on a sewage farm. Dr. Russel was kind enough to send me samples of sick soils from three different sources, and of these I have devoted most of my attention to the sample derived from a sick tomato bed.

For the purpose of separation of the organisms I used various types of agar-plate, and as much still remains to be done from the cultural point of view, I have decided to publish the full details of my methods in the later paper. It must, however, be evident that the attempt to solve the problem of how far any protozoon is really doing damage under the normal soil conditions from a study of the forms grown on agar-plates must be a very difficult and dangerous one. It is more than probable that the sample of population cultivated under all the present methods may not by any means be a fair representation of the population of the given soil. On the other hand, it is, I think, important to observe that one finds that each small sample of a given soil for each culture-condition has given rise to a fairly constant specific fauna, whereas samples on other soils under these conditions have also given rise to constant but quite distinct faunas. If these facts are taken into consideration, it seems hard to believe that all these forms are a purely negligible factor in the question of soil-sickness, since it is important to note that many of these forms are new and—a point of difference from the ciliates—have never previously been found in fresh-water pools or infusions. It is, therefore, unlikely that all these organisms are present in the soil as cysts, because they must presumably lead a trophic life somewhere, and it seems probable that their trophic activity is a cause of soil-sickness. On the other hand, it is quite possible that the form or forms which are most dangerous in cases of soil-sickness may not up to the present have survived under the conditions of plate-cultures. I do not think that this view is probable, but the question can, of course, only be decided by parallel cultures of three kinds: (1) Plants grown on the sterilised soil; (2) plants grown on the sterilised soil to which pure cultures of the various protozoa, with the bacteria on which they feed, have been added; and (3) plants grown on the sterilised soil to which cultures of the protozoa without the bacteria have been added.

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* 'Roy. Soc. Proc.,' 1911, B, vol. 84, p. 165.

As regards methods of preparation, wet cover-slip smears were solely used. In this connection a device suggested to me by Mr. Jamieson, of the Natural History Department, Glasgow University, has proved extremely useful. It is obvious that with a large number of cultures on one's hands a number of smears must be fixed which cannot be immediately stained. The smears in these cases after fixation were stacked in tubes in 70-per-cent. alchol. It was found, however, in some cases, after the smears had been some time in the tubes, that in the case of thin smears it was extraordinarily hard to tell the right from the wrong side of the cover-slip. I therefore got some oblong cover-slips of which one corner was cut off, made by Messrs. Frazer, of Edinburgh. It will, I think, be obvious that, if one makes up one's mind to always have the smear on the lower surface of the cover-slip when the top right-hand corner is cut, no mistake of this kind can possibly arise.

I should like to take this opportunity of thanking Dr. Russel for his kindness in sending me samples of soil, and for much advice, and also

Prof. Minchin for allowing me to write up these notes in his laboratory at the Lister Institute.

Of the forms which I cultivated from the sick tomato-soil a rough list will be found at the end of the paper. In this note I wish to describe a flagellate which passes through its life-cycle in my cultures, terminating in the formation of a zygote-cyst,* with great regularity. I do not propose to enter into the question of the nomenclature of this form in any detail here. It appears to me to be identical with the flagellate figured by Stein as *Cercomonas termo*, which he identifies with *Monas termo* of Ehrenberg, and which is therefore presumably identical with the *Oikomonas termo* of Bütschli. The trophic form of this organism is a roughly spherical animal, provided at its physiologically anterior end with a single, rather short, thick flagellum (Plate 10, fig. 1).

The size of the animal may be somewhat variable. In young cultures, in which no gametes occur, the active forms seem to measure about $4 \cdot 5 \, \mu$, whereas in the older cultures larger forms are met with. The body, particularly at its posterior end, contains large rounded vacuoles. These vacuoles may disappear, and are probably mostly food-vacuoles, and I cannot find any evidence for the existence of a true contractile vacuole. In life, the posterior end of the body may undergo amœboid movement, and, more particularly in actively swimming forms, the body may assume a rather more elongated shape than in the resting form (fig. 2). The body is often found crowded with ingested bacteria, and a number of small granules which appear to stain reddish in neutral red. In life, a nucleus, with a fairly large caryosome, can be distinguished clearly. In stained forms the flagellum can be seen to arise from quite a large blepharoplast, which stains vividly in iron-hæmatoxylin. The nucleus in the stained preparations is large and vesicular. Most of the chromatin appears to be contained in the caryosome, but a certain amount seems to be present on the nuclear wall (fig. 1).

The division in this monad seems to be initiated by the division of the blepharoplast (fig. 3), but this is quickly followed, or possibly in some cases preceded, by the splitting of the flagella. Up to the present I have found no evidence for the outgtowth of new flagella in the dividing forms of this animal. The nucleus up to this stage presents its characteristic appearance of a dark caryosome lying in a large nuclear space. It quickly becomes much darker, and the whole space is crowded with relatively large chromatin granules (fig. 4). A curious feature in this division is the apparent increase in the amount of chromatin in the nucleus during the early stages of division, an increase which it seems difficult to account for when one considers the amount of chromatin in the caryosome and on the wall of the resting nucleus (fig. 5). One of the division-products of the blepharoplast, with its flagellum, now passes around the nucleus, being still connected with the other blepharoplast by a faintly staining strand, which seems to have more affinity for eosin than for chromatin stains (*vide* figs. 5 and 6).

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* It is unfortunately impossible at present to prove conclusively that this process is not an autogamy, since up to the present I have not carried on continuous observations on conjugating forms in life.

The chromatin granules are now massed in an irregular oval body on the strand connecting the two blepharoplasts, as is shown in figs. 5 and 6. The chromatin masses then appear to stream in an irregular manner towards either blepharoplast. There they become collected into two crescent-shaped masses, in the convexity of each of which a blepharoplast lies (fig. 7). At the same time the body of the flagellate becomes much elongated in the axis of the line joining the two blepharoplasts. About this stage there seems to be mysterious diminution in the amount of chromatin comparable to the increase which was noted in the early stages of division. The body of the flagellate now becomes constricted (fig. 8) and the division-products separate (fig. 9). It seems probable, from the relative frequency with which the stages are met on the stained films, that the early stages of division are much slower than the later.

Before proceeding to a description of the process of encystation in this flagellate, I should like to draw attention to some features in the behaviour of the cultures with regard to the life-cycle of this animal. In the first place, the appearance of an encystation-epidemic in any particular culture seems to be primarily due to the amount of moisture on the culture-plate. I have, on several occasions, started series of three parallel cultures: (*a*) rather dry, (*b*) fairly moist, and (*c*) quite moist. In all three the organisms would do well at a temperature of about 18° C., with the result that at the end of the third day the fixed films of any culture would show a large number of division-figures. An encystation-epidemic would, however, set in in (*a*) at the end of a week, in (*b*) at the end of about 12 days, whereas cultures of the type of (*c*) have been kept over two months without the appearance of encystation.

What the ultimate factor in this appearance of encystation-epidemics in drying cultures may be I should not at present care to hazard an opinion. Possibly it may be simply a direct osmotic effect upon the animal, possibly it may introduce changes in the bacterial flora of the plate resulting in a diminution of the appropriate food-supply of the animal, or possibly it may initiate the formation of some substance by the bacteria toxic to the flagellate and to which it reacts by encystation. But it is evidently useless attempting to analyse the physiology of this phenomenon without a great deal more experimental evidence than I have at present collected.

I will only draw attention to one other feature in the physiology of these cultures before proceeding with my account of the morphological features presented in the formation of the zygote-cyst. On several occasions I have attempted to make sub-cultures of this flagellate from flourishing cultures in which few or no cysts were present. These cultures always failed. Whether this failure was primarily due to a zoological or a bacteriological factor I am at present unable to say, but sub-cultures made from the same primary cultures at a later stage when the cysts had been formed were uniformly successful.

The conjugation in this flagellate is probably isogamous, and there seems to be a certain amount of evidence tending to show that the gametes are characterised by a slightly larger size of body and by a slightly smaller nucleus containing a relatively smaller caryosome. The gametes usually contain quantities of ingested bacteria, and it would seem that this pronounced trophic activity constantly precedes conjugation. The gametes seem to come into contact laterally, and the zygote formed by their fusion swims around actively for some time by means of its two flagella (figs. 10 and 11), which seem to be always inserted near the same pole. The zygote now grows in size, the growth appearing to be largely due to the formation of a large vacuole in the posterior portion of the body. At a slightly later stage this vacuole is found to be occupied by a solid round body, which I am inclined to regard as a reserve-body (figs. 13, 14). The flagella now disappear, and I have not yet succeeded in tracing the fate of the blepharoplasts in the later stages of the zygote.

The zygote now secretes a wall, and presents a very characteristic appearance with its two nuclei closely applied to the anterior wall of the large vacuole containing the reserve-body. The reserve-body is best shown in early cysts stained with iron-hæmatoxylin, which stains it a faint yellowish colour. The ripe cysts can only be stained with great difficulty with acid hæmatoxylin. In these permanent preparations the reserve-body cannot be seen (figs. 15, 16). The nuclei apparently now undergo some process of reduction division, as is shown in figs. 13 and 14. But I hope to return to this point in my final paper. The ripe zygote-cyst (*vide* fig. 16) contains a single nucleus, the result of the fusion of the nuclei of the two gametes. To the question of some of the peculiar features displayed by this life-cycle, and the light which they may throw on the affinities of this flagellate, I hope to return in my forthcoming paper.

In addition to these monads I have succeeded in obtaining pure animal cultures of a number of other forms. Amongst these some have been previously described by other workers, and I give a short list of the forms that I have up to the present identified :—

(1) *Chlamydophrys stercorea ;* (2) *Amœba diploidea ;* (3) *Amœba sp. ?* (4) *Amœba sp. ?* (5) *Amœba sp. ?* (6) *Copromonas ;* (7) *Bodo sp. ?* (8) *Prowazekia sp. ?* (9) *Astasia sp. ?*

To some of these forms I hope to return in my later paper, but I should like to point out that I have now worked for over a year with cultures of a form which seems to me absolutely identical with *Chlamydophrys stercorea,* and have entirely failed to find any indication in confirmation of Schaudinn's account of the sexual stages of this form. This is the more difficult to understand as the account and the figures which he has published of the asexual stages are in complete accord with those I find in my cultures. Schaudinn, as will be remembered, stated* that in all his cultures, at the end of a few days, a formation of flagellate gametes set in, which after an isogamous conjugation gave rise to small zygote-cysts. These cysts, according to his account, could only be further cultured after the passage through the gut of some vertebrate. My cultures, under a large number of different conditions, have uniformly ended in a cyst formation without any sexual process, and these cysts could always be readily cultured on all fresh media, and would not pass in a living condition through the gut of a fowl. I have found flagellates in some of my early *Chlamydophrys*-cultures, but I hope to be able to show in my forthcoming paper that these are parasites, and have no connection with the life-cycle of *Chlamydophrys.*

EXPLANATION OF PLATE.

All the figures were drawn with a camera lucida under Zeiss comp. oc. 18 and 1·5 apochr. objective.

All the figures except 1, 2, 11, and 12 were drawn at table level.

1. Normal round form of *Monas termo.*
2. Active elongate form.
3. Early stage of division ; the flagella and blepharoplasts have divided.
4. Slightly later stage of division ; the blepharoplasts with their attached flagella are beginning to separate, and the nucleus is filled with a number of spherical masses of chromatin.
5. The blepharoplasts with their attached flagella have moved apart so that the enlarged nucleus filled with the chromatin-spheres lies between them. The blepharoplasts are still united by a thread which stains faintly with eosin.

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* "Untersuchungen über die Fortpflanzung einiger Rhizopoden," 'Arb. a. d. kais. Gesundheitsamt,' 1903, vol. 19, p. 547

6. Later stage of division; the chromatin-spheres are massed on the thread joining the blepharoplasts.
7. The chromatin-masses have passed to either pole of the dividing monad; the line joining the blepharoplasts still persists.
8. Division is almost complete, and the line between the blepharoplasts has disappeared.
9. One of the products of a recent division; the nucleus has not yet been reconstructed.
10. Early stage of conjugation.
11. Active zygote-stage showing the approximation of the blepharoplasts and large vacuoles.
12. Early stage of encystation of the zygote; the wall at this stage is quite soft and stains with eosin.
13. Unripe cyst, showing reserve-body. Iron-hæmatoxylin preparation.
14. Unripe cyst, showing possibly reduction-division on the part of the nuclei.
15. Almost ripe zygote cyst, with elongated synkaryon. Hæmalum-preparation; does not show reserve-body.
16. Ripe cyst, with round synkaryon. Hæmalum-preparation; does not show reserve-body.

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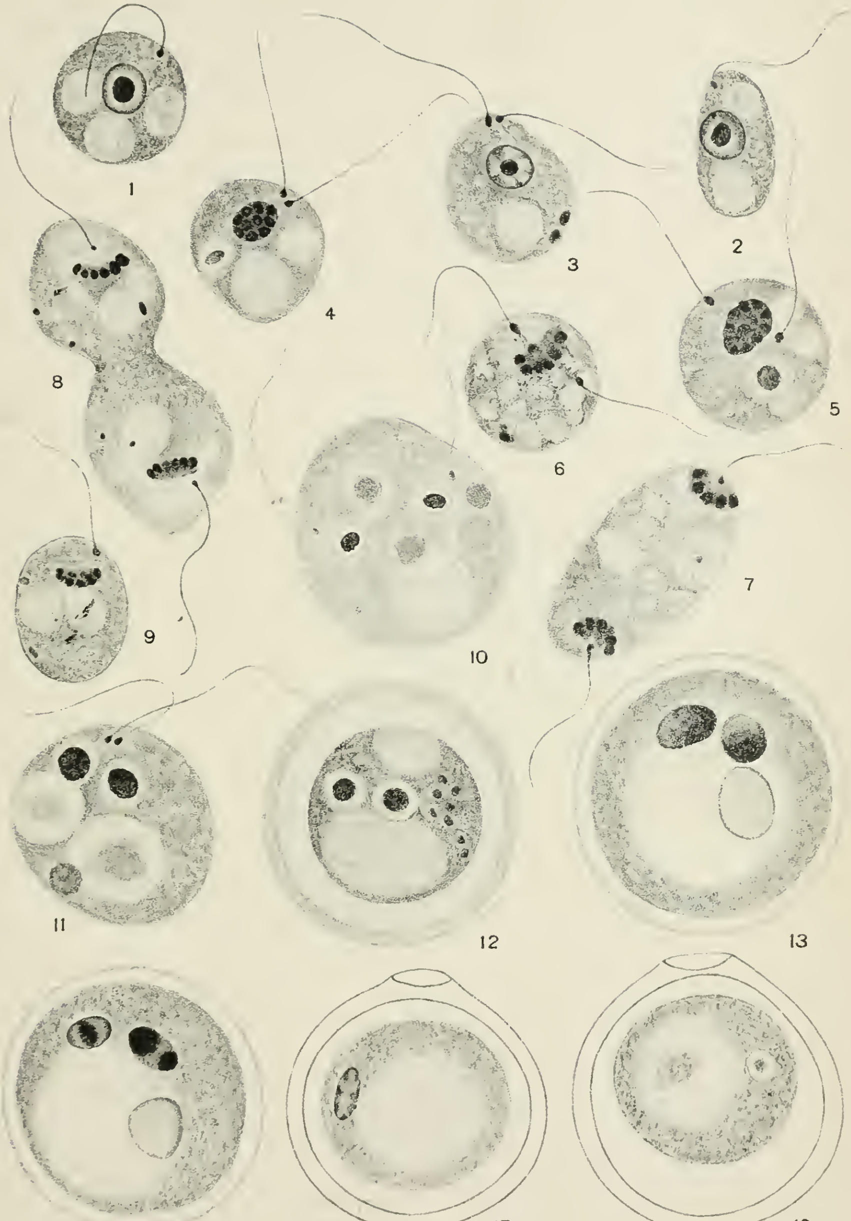
Martin. *Roy. Soc. Proc.* B. Vol. 85 *Pl. 10.*



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THE INFLUENCE OF THE CHEMICAL CONSTITUTION OF CERTAIN ORGANIC HYDROXYL AND AMINIC DERIVATIVES ON THEIR GERMICIDAL POWER

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The method employed in estimating the germicidal powers of various organic substances was that devised by Martin and Chick (*Journ. of Hygiene*, 1908, **8**, No. 5. Nov. p. 654); it consisted in making a comparison of the concentrations of the substance and of pure phenol required to kill an equal number of organisms of the same species in a constant volume of the disinfectant solution (5 cc) during a constant period of time (15 minutes) and at a constant temperature (20° C).

All test-tubes, pipettes and flasks were first sterilized and different amounts of a standard solution of the substance under examination were introduced into a series of test-tubes, and sterilized water was then added so as to make up each volume to 5 cc. so that the tubes contained a constant volume of solutions containing different concentrations of the substances. A series of phenol solutions was similarly prepared and the two sets of tubes were immersed in a thermostat at 20°. When the tubes had attained this temperature five drops were added to the first dilution from a standard capillary pipette (1 drop = 0.02cc) of a 24 hours' culture of the organism, obtained by inoculating 6 cc. of broth with a standard loopful of agar culture.

The inoculation of the disinfectant solutions proceeded at one minute intervals and at the 15th minute two tubes containing 10 cc. of glucose broth were each inoculated with two loopfuls of the contents of the first tube taken out by means of a standard platinum loop (a loopful of broth weighing about 0.004 gram). The other tubes of the reacting solutions were also subcultured at one minute intervals so that in each case the disinfectant acted for 15 minutes.

The subculture tubes were inoculated at 37° for 96 hours and then examined when the presence or absence of growth was referred to the corresponding dilution. The average of the lowest concentration of the substance which killed and the highest concentration which failed to kill was compared with the corresponding mean phenol concentration and the latter divided by the former gave the carbolic acid co-efficient of the substance.

The organisms used throughout the work were *Staphylococcus pyogenes aureus* and *Bacillus typhosus.* The broth was made according to the following recipe: Brand's meat juice 10 cc. salt 5 grams, peptone 10 grams, glucose 10 grams in 1 litre of tap water. The reaction of the broth was kept constant being + 6 to + 7 to phenolphthalein (Eyre's notation).

The important difference between this method of standardizing disinfectants and the Rideal-Walker method was the selection in the former of a constant reacting time for the germicides, namely 15 minutes.

I. COMPOUNDS CONTAINING HYDROXYL GROUPS

1. *The aliphatic alcohols.*

Jalan de la Croix (*Archiv. f. exp. Pathol.* 1881, p. 175) found that a 1 in 21 aqueous solution of ethyl alcohol prevented the growth of bacteria in broth, but 22 p.c. solutions were required to kill them. Stronger solutions of alcohol (83 p.c.) were necessary to kill spores.

Koch (*Mittheil. a. d. K. Gesundh.* 1881, Vol. 1) found that anthrax spores were not killed by immersion for 110 days in absolute alcohol and in its 33 p.c. and 50 p.c. dilutions. A 1 p.c. solution impeded and an 8 p.c. solution completely arrested the development of anthrax spores.

Fowler (*Journal of the Royal Army Medical Corps.* 1907, July, "Some disinfectant values") found that ethyl alcohol possesses a carbolic acid coefficient of only 0.03 when tested on *B. typhosus.*

There is evidence, therefore, that alcohol possesses feeble germicidal properties. Nevertheless it has sometimes been employed for antiseptic purposes in surgery, but its chief value is as a vehicle for the application of other therapeutic substances.

All the following alcohols, with the exception of amyl alcohol are freely miscible with water. The carbolic acid coefficients of these compounds were determined on *Staphylococcus py. aureus* in the absence of organic matter.

Alcohol	Concentration required to kill in 15 minutes (parts per 1,000)		Carbolic acid coefficients
	Alcohol	Phenol	
Methyl alcohol	350	9.0	0.025
Ethyl alcohol	350	9.5	0.027
Ethyl alcohol (with *B. typhosus*)	325	8.5	0.026
Propyl alcohol	140	9.0	0.064
iso-Propyl alcohol	210	8.5	0.040
n-Butyl alcohol	41	9.5	0.250
Trimethylcarbinol	190	10.5	0.055

iso-Amyl alcohol (a saturated ($\frac{1}{2}\%$) solution at 20° failed to kill in 15 minutes).

The germicidal powers of the foregoing alcohols are considerably less than that of phenol so that the action of the latter must be largely determined by its benzene nucleus. The equality in germicidal powers of methyl and ethyl alcohols is an exception to the general tendency for this action to increase as the homologous series is ascended. This abnormality in the case of methyl alcohol corresponds with the anomalies observed in regard to its chemical and physical properties.

A comparison of the results obtained with *n*-butyl alcohol and trimethylcarbinol shows that the primary alcohol is much superior to the isomeric tertiary alcohol in germicidal power.

Normal propyl alcohol also exceeds its isomeride, *iso*propyl alcohol in germicidal power.

It will be noticed that in the foregoing series of alcohols those members having the higher specific gravity, heat of combustion and boiling point have the greater germicidal power. The phenomenon of germicidal action may be determined by those constitutive influences which determine the physical and chemical properties of substances.

2. THE PHENOLS AND THEIR HOMOLOGUES AND DERIVATIVES

Alcohol is sometimes employed for dissolving the phenols so as to obtain them in a form suitable for disinfecting and antiseptic purposes. For example, there are two non-official preparations of thymol containing ethyl alcohol, liquor antisepticus, and liquor thymol. Kronig and Paul (*Zeitsch.* fur. *Hygiene* 1897, 25, 2, p. 1.) showed that alcohol decreased considerably the germicidal action of phenol on anthrax spores and a solution of phenol in absolute alcohol had very little germicidal power. This inhibiting effect is probably due to the greater solubility of phenol in alcohol, which alters the distribution of this germicide between water and the bacterial proteins with the result that the spores absorb considerably less of the phenol.

In practice the destruction of non-sporing pathogenic organisms is generally the object in view, and it is important to know what effect alcohol has on the germicidal action of phenols on such organisms. Non-sporing microorganisms are more sensitive to the germicidal action of alcohol than are spores, and it was accordingly of interest to ascertain how far the contribution of alcohol towards the bactericidal action of the phenols counterbalanced the inhibiting influence of alcohol on the partition coefficient.

In investigating the effect of known percentages of alcohol on the germicidal action of phenol the experiments were carried out in the manner already described, except that the various dilutions of phenol were made up with sufficient alcohol to bring the percentage of this solvent to the desired extent. The large error involved in the bacteriological test obviated any necessity for a correction for the contraction in volume produced by mixing water and alcohol.

The following table gives the concentrations of phenol required to kill a constant number of *B. typhosus* or *Staphylococci* in 15 minutes at 20° in the presence of various concentrations of alcohol.

Disinfectant	Organism		Percentage of Alcohol				
		0 water	5	10	15	20	30
Phenol in 1000 parts of solvent	*B. typhosus*	8.5 mean of 4 expts.	8.5	7.0 mean of 2 expts.	6.5	4.75 mean of 3 expts.	1.4 mean of 3 expts.
Phenol in 1000 parts of solvent	*Staphylococcus*	9.5		7.5			

The action of the alcohol on the above non-sporing organisms was sufficiently appreciable to overcome within 15 minutes its depreciating effect on the action of the phenol, so that the presence of this solvent led to an apparent increase in the germicidal efficacy of the phenol, a 0.15 p. c. solution of phenol in 30 p.c. alcohol doing the same amount of disinfection as a 0.85 per cent. aqueous solution. The changes produced by alcohol on the germicidal efficiency of resorcinol and thymol were next investigated.

ORGANISM. *B. Typhosus*

Disinfectant	In Water		In 30 p.c. Alcohol	
	Concentration Killing in 15 mins. at 20°	Carbolic Acid Coefficient	Concentration Killing in 15 mins. at 20°	Carbolic Acid Coefficient
Phenol in 1000 parts solvent	8.5	1	1.1	7.7
Resorcinol in 1000 parts solvent	28.0	0.3	5.0	0.22
Phenol in 1000 parts solvent	7.5	1	1.3	5.6
Thymol in 1000 parts solvent	0.325	23	0.18	7.2

Although 30 p.c. alcohol increases considerably the apparent germicidal effect of resorcinol and thymol on *B. typhosus*, yet as the increase is less marked than in the case of phenol, the carbolic acid coefficients of these two substances were reduced by the presence of alcohol of this concentration. Although the use of alcohol cannot be recommended for solutions of phenols required to destroy pathogenic sporing organisms yet this solvent may be employed with advantage in the disinfection of non-sporing organisms, particularly as the phenols are less caustic in alcoholic than in aqueous solution.

As alcohol affects the germicidal powers of different substances to a varying extent, different conclusions may be reached with regard to their efficacy, depending on the medium employed in the process of disinfection, and these irregular variations should be borne in mind in considering the possible relationship between chemical constitution and germicidal power.

INFLUENCE OF ORIENTATION ON THE GERMICIDAL ACTION OF THE DIHYDROXYBENZENES

ORGANISM—*B. Typhosus.* Temperature 20°

Dihydroxybenzene	Concentration of substance required to kill in 15 minutes $x/1000$	Concentration of phenol required to kill in 15 minutes $x/1000$	Carbolic Acid Coefficient
Resorcinol	26.0	7.50	0.29
Catechol	17.0	8.25	0.48
Quinol	7.5	8.50	1.1

The minimum effect is produced by the meta-isomeride and the maximum by the para-compound, the ortho-derivative giving an intermediate value of the carbolic coefficient. It is of interest to note that the two isomerides containing hydroxyl groups in sympathetic positions (ortho and para) exert a greater germicidal action than resorcinol in which these groups are in the apathetic meta-position with respect to each other.

THE NITROPHENOLS

The only nitrophenols which appear to have been previously employed as germicides are picric acid (2:4:6—trinitrophenol) and the potassium salt of dinitro-*o*-cresol [$C_6H_2(No_2)_2(CH_3)$ OK.]

Jalan de la Croix (*Arch. f. expt. Pathol.* Jan. 27, 1881) found that 1 in 1000 aqueous solutions of picric acid killed bacteria in infusions of egg-white. Cheron (*J. de Therapeut, Gubler*, 1880, p. 121) used a saturated solution of this compound for purposes of disinfection in hospitals. Koch (*l.c.*) found that 1 in 10,000 dilutions of picric acid impeded the development of anthrax spores, but that 1 in 20,000 dilutions were not sufficient to arrest growth.

Potassium diunitro-*o*-cresoxide has been employed chiefly as an insecticide and a fungicide.

The nitrated derivatives of phenol are not very soluble in cold water but solution was facilitated by gentle warming.

ORGANISM. *Staphylococcus py. aur.* Temperature 20°

Substance	Concentration of substance killing in 15 minutes $x/1000$	Concentration of phenol killing in 15 minutes $x/1000$	Carbolic acid coefficient
p-Nitrophenol	4.6	10.5	2.3
Potassium *p*-nitrophenoxide	20.0	10.5	0.52
m-Nitrophenol	2.7	9.5	3.5
Picric acid	1.4	10.5	7.5
Picric acid with *B. Typhosus*	1.0	8.5	8.5

Potassium *p*.nitrophenoxide contained 2 H_2O of crystallization, and allowing for this the carbolic acid coefficient of dry salt, $No_2.\ C_6\ H_4.\ O\ K = 0.52 \text{ x } {}^{213}/_{177} = 0.62$.

The successive introduction of nitro-groups into the phenol molecule produces a progressive increase in the germicidal power of the substance but the practical application of these nitro-compounds is hindered by their poisonous and staining properties. In this series the meta-isomeride is a more powerful germicide than the para-compound. The alkali salt is far less efficacious than the free nitro-derivative, this influence of salt formation will be plainly noticeable in the following series of aromatic hydroxycarboxylic acids.

The following table gives a comparison of the carbolic acid coefficients of the nitrophenols and cresols, the organism employed being *Staphylococcus*.

Nitrophenols	Coefficient	Cresols	Coefficient
1 : 2	—	1 : 2	2.1
1 : 3	3.5	1 : 3	2.0
1 : 4	2.3	1 : 4	2.4

Although *p*-cresol and *p*-nitrophenol have practically the same bactericidal power, *m*-nitrophenol is considerably more active than *m*-cresol.

Saturated solutions of *o*-nitrophenol (0.3 p.c.) and of 2 : 4-dinitrophenol (0.1 p.c.) failed under the prescribed experimental conditions to kill *Staphylococcus py. aur.* in 15 minutes.

COUMARIN, THE COUMARIC ACIDS AND THEIR ALKALI SALTS

The three coumaric acids (hydroxycinnamic acids) combine in their molecular structure the chemical constitutions of cinnamic and salicylic acids and have accordingly been suggested as substitutes for the latter acids in the therapeutic application of these substances. (*British Medical Journal*, 1905, *i.* 1143). The acids have been employed for this purpose in the form of their sodium salts, which are freely soluble in water. The salt of the ortho-acid appeared to be more physiologically active than that of the para-acid whereas the salt of the meta-acid, exerted a more powerful action than either of these substances.

Coumarin is so sparingly soluble in cold water that it was for the purpose of the test, converted into its soluble sodium salt; in these circumstances it dissolves in aqueous sodium hydroxide, forming sodium coumarinate, the *cis*-isomeride of sodium *o*-coumarate which has the *trans*-configuration.

ORGANISM: *B. Typhosus* (24 *hours' culture*)

Substance	Concentration of substance killing in 10 minutes	Concentration of phenol killing in 10 minutes	Carbolic acid coefficient
Sodium *o*-coumarate	1 in 10	1 in 105	0.095
Sodium *o*-coumarinate	1 in 10 failed to kill in 10 minutes		
Sodium *p*-coumarate	1 in 10 failed to kill in 10 minutes		

The results show that the germicidal action of these sodium salts is very feeble, the ortho-coumarate being the most active. This feeble action of soluble alkali derivatives of aromatic phenolic compounds is noticeable in the foregoing case of potassium *p*-nitrophenoxide.

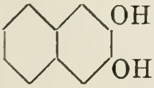
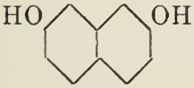
o-Coumaric acid and coumarin are freely soluble in 30 p.c. alcohol and their germicidal action was compared with that of phenol in the same medium.

Substance	Organism	Concentration of substance required to kill in 10 minutes	Concentration of phenol required to kill in 10 minutes	Carbolic acid co-efficient
o-Coumaric acid	*B. coli.*	1 in 500	1 in 620	0.80
o-Coumaric acid	*B. typhosus.*	1 in 500	1 in 680	0.73
Coumarin	*B. typhosus.*	1 in 550	1 in 620	0.56

THE DIHYDROXYNAPHTHALENES

Although B-naphthol and certain of its sulphonic acids have found employment as germicides the effect of the dihydric naphthols on pathogenic organisms has not hitherto been examined.

1 : 5-Dihydroxynaphthalene is so sparingly soluble in water that its action could not be ascertained in aqueous solution. Two of the 3 isomerides having both their hydroxyl groups in B-positions, namely 2 : 3-dihydroxynaphthalene (m.p. 161°) and 2 : 7-dihydroxynaphthalene (m.p. 190°) were taken for the test, these compounds being sufficiently soluble in cold water, and, as in the foregoing experiments, the carbolic acid coefficient was determined in the absence of added organic matter, *B. typhosus* being taken as the test organism.

Dihydroxy-naphthalene	Concentration of the substance killing in 15 minutes	Concentration of phenol killing in 15 minutes	Carbolic acid coefficient
	1.9 in 1000	8.5 in 1000	4.4
	2.8 in 1000	8.0 in 1000	2.8

The isomeride containing the hydroxyl groups in contiguous positions is the more active, and both compounds are greatly superior to phenol in bactericidal power.

The dihydroxynaphthalenes have not hitherto been suggested for germicidal purposes, and although the cost of preparing the 2 : 3-isomeride would militate against its employment in this direction yet the 2 : 7-isomeride has been prepared economically on a manufacturing scale as an intermediate product in the formation of organic coloring matters. Naphthalene is sulphonated with 5-6 parts of concentrated sulphuric acid for 4 hours at 140°; the resulting naphthalenedisulphonic acid is converted successively into its calcium and sodium salts. The latter is fused with 2 parts of sodium hydroxide and 0.5 part of water, the melt is acidified with dilute sulphuric acid, and, after expelling the sulphur dioxide by means of wet steam, the solution is cooled when 2 : 7-dihydroxynaphthalene separates in almost colorless crystals and is purified by further crystallization from hot water.

It will be noticed that the two dihydroxynaphthalenes examined above greatly exceed the three dihydroxybenzenes in their germicidal action.

II. THE ORGANIC AMINES

1. *Aliphatic Amines*

Koch (*l.c.*) found that 5 p.c. solutions of trimethylamine in water did not kill anthrax spores in 12 days.

The mixture of aliphatic amines from the interaction of herring-brine and lime has been used for the sterilization of sewage, for which purpose Klein stated that it was very efficient. It consists largely of trimethylamine and under the name of " Aminol " has been introduced as a general disinfectant.

The aliphatic amines used were all freely soluble in cold water. Their germicidal powers determined with *B. Typhosus* are given below.

Amine	Concentration of amine killing in 15 minutes	Concentration of phenol killing in 15 minutes	Carbolic coefficient
Ethylamine	7.09 in 1000	9 in 1000	1.27
Ethylene-diamine	Between 2 p.c. and 30 p.c.	8.5 in 1000	Between .03 & .4
iso-Amylamine	3 in 1000	8.5 in 1000	2.8
n-Heptylamine	0.35 in 1000	8.5 in 1000	24.3

The aliphatic amines therefore possess considerable germicidal power which increases with the size of the alkyl group in the amine molecule and which, when *n*-heptylamine is reached, attains a very high value.

The apparently high germicidal power was found *not* to be due to the inhibitory effect of the traces of amine carried over into the sub-culture tubes, as when the contents of the broth-tubes were sub-cultured into a second series of tubes in which the concentration of the amine did not exceed in any experiment .00000025 p.c., the carbolic coefficient was not affected.

Ethylenediamine is much feebler than ethylamine in germicidal power.

The germicidal powers of the fatty amines are compared in the following table with those of the corresponding alcohols:

ORGANISM. *B. Typhosus*

Amine	Coefficient	Alcohol	Coefficient
Ethylamine	1.27	Ethyl alcohol	0.026
iso-Amylamine	2.80	*iso*-Amyl alcohol	Under 1.7

The aliphatic amines are therefore considerably superior in germicidal power to the corresponding alcohols.

2. *Aromatic Amines*

Angus Smith (*Disinfectants*, Edinburgh, 1869) examined the germicidal power of aniline and regarded it as a disinfectant of moderate efficiency.

Fischer (*Mittheil a.d. K. Gesundt.* Vol. II) has found that a solution of aniline in water disinfected tubercular sputa in 24 hours.

Many of the aniline dyes have been shown to possess inhibitory and germicidal powers. Stilling (*Lancet*, 1890, Vol. XI. p. 965) showed that dilutions of 1 in 500 to 1 in 1000 of the methyl-violets prevented the growth of moulds on bread and 2 in 1000 dilutions prevented the souring of milk.

Prioux (*Internat. J. of Microscopy, and Nat. Science*, Vol. III, part 18) showed that 1 in 500 to 1 in 2000 dilutions of the methyl violets arrested the growths of *B. typhosus* and *B. coli*.

Fowler (*l.c.*) determined the germicidal power of methylene-blue on *B. typhosus* and found that it possessed a carbolic acid coefficient of 1.5.

Pyridine has been used mixed with oil of peppermint in the treatment of diphtheria and the injection of its aqueous solutions has been beneficial in gonorrhoea (Helbeig, *Mod. Mat. Med.* p. 65). Blyth showed that *Staphylococcus py. aur.* was killed by 1 p.c. solutions of pyridine and its homologues derived from bone-oil.

May found that magenta base (consisting largely of rosaniline) exceeds phenol in germicidal power and is moreover less toxic and more readily diffusible (*J. Amer. Medical Association*, 1912, 8 (16) April 20th).

With the exception of pyridine, the aromatic amines used were not freely soluble in water but solution was accelerated by gentle warming.

ORGANISM. *B. Typhosus*

Substance	Concentration of substance killing in 15 minutes	Concentration of phenol killing in 15 minutes	Carbolic Acid Coefficient
Aniline	15 in 1000	8.5 in 1000	0.57
o-Toluidine	7.5 in 1000	7.5 in 1000	1.00
m-Toluidine	6.5 in 1000	8.5 in 1000	1.30
p-Toluidine	6.0 in 1000	7.5 in 1000	1.25
Pyridine	48 in 1000	8.5 in 1000	0.18
ac-Tetrahydro-*B*-napthylamine	1.6 in 1000	8.5 in 1000	5.3
o-Phenylenediamine	a saturated solution (2 p.c.) failed to kill in 15 minutes		under 0.42
m-Phenylenediamine	a 4 p.c. solution failed to kill in 15 minutes		under 0.2
p-Phenylenediamine	a saturated solution (3 p.c.) failed to kill in 15 minutes		under 0.3
Tolylene-1:4-diamine	a 4 p.c. solution failed to kill in 15 minutes		under 0.2

The germicidal power of aniline is therefore considerably less than that of phenol. The toluidines exceed aniline in germicidal power, indicating that the introduction of a methyl group into the benzene nucleus of aniline increases bactericidal action. The relative positions of the amine—and methyl—groups has some effect on germicidal power, which is greatest when the groups are in the meta- or para-position to one another.

Of all the monacidic amines examined, whether aliphatic or aromatic, pyridine is the feeblest in germicidal action.

The results obtained with aniline and phenylenediamines and with the toluidines and tolylene—2.4-diamine show that the entrance of a second amino-group into the benzene nuclei of aniline and the toluidines leads to considerable decrease in germicidal power. The aliphatic diamine, ethylene-diamine, is also weaker than ethylamine in bactericidal power.

In the following table the germicidal efficiencies of the toluidines and cresols are compared.

ORGANISM. *B. Typhosus*

Toluidines	Carbolic Coefficient	Cresols	Carbolic coefficient
Ortho.	1.00	Ortho.	2.6
Meta.	1.30	Meta.	2.6
Para.	1.25	Para.	2.6

The cresols are therefore consistently superior to the toluidines in germicidal power.

In the following table the germicidal powers of the dihydroxybenzenes and diaminobenzenes are compared.

ORGANISM. *B. Typhosus*

Amines	Coefficient	Phenols	Coefficient
o-phenylenediamine	Under 0.42	Catechol (1 : 2)	0.48
m-phenylenediamine	Under 0.2	Resorcinol(1 : 3)	0.29
p-phenylenediamine	Under 0.3	Quinol (1 : 4)	1.1

The dihydroxybenzenes are therefore superior to the diaminobenzenes in germicidal power.

The superiority in germicidal efficiency of phenol to aniline, of the cresols to the toluidines and of the dihydroxybenzenes to the diaminobenzenes indicates that the substitution of the amino-group for the hydroxy-group in the benzene nucleus is accompanied by a decrease in germicidal power. In the aliphatic series, on the other hand, the substitution of an amino-group for the hydroxy-group leads to a great rise in germicidal efficiency.

The germicidal powers of the aliphatic and aromatic amines are compared below.

ORGANISM. *B. Typhosus*

Amine	Carbolic Coefficient
Ethylamine	1.27
*iso*Amylamine	2.8
n-Heptylamine	24.3
ac-Tetrahydro-β.naphthylamine	5.3
Aniline	0.57
o-, *m*-, & *b*-Toluidines	1.00; 1.30; 1.25
Pyridine	0.18

It is seen that ethylamine exceeds aniline and is approximately equivalent to the toluidines in germicidal power whilst *iso*amylamine and *n*-heptylamine surpass very considerably the aromatic amines in efficiency. High germicidal power in the amines thus seems to be produced not by the presence of groups of an acidic nature such as phenyl and tolyl but by the presence of alkyl groups on which the strong basic properties of the aliphatic amines largely depend.

It is possible that the fatty amines owe their high germicidal efficiency in aqueous solution partially to the presence of hydroxyl ions liberated through ionisation of the alkylammonium hydroxides which are formed by the combination of the amines with water—

$$C_2H_5\cdot NH_2 + H_2O = C_2H_5\cdot NH_3\cdot OH$$

$$C_2H_5\cdot NH_3\cdot OH = C_2H_5\cdot \overset{+}{NH_3} + \overset{-}{OH}$$

From this point of view, however, the feeble germicidal action of the strong base, ethylenediamine is certainly exceptional.

ac.—Tetrahydro-β-naphthylamine has a high carbolic acid coefficient which is noteworthy in connection with the chemical relationships of this amine. Its amino-group is attached to a fully hydrogenized ring; the substance is a strong base, non-diazotisable, absorbing carbon dioxide from the air and soluble in water. The high germicidal power of the base is also of interest when considered in conjunction with the powerful fever-inducing action of this substance on the higher animals. The replacement of hydrogen in ammonia by radicles of an acidic nature such as phenyl or tolyl, gives rise to substances of feeble germicidal action. Other acidic groups such as succinyl have a similar effect. A 2 p.c. solution of succinimide failed to kill *B. typhosus* in 15 minutes, thus indicating a carbolic acid coefficient of less than 0.4.

THE EFFECT OF AGE ON THE GERMICIDAL POWER OF AROMATIC AMINES

Most of the aromatic amines gradually become highly colored on exposure and the following comparison has been made of the germicidal powers of aniline and *o*-toluidine in the colored and colorless (redistilled) condition.

ORGANISM. *B. Typhosus*

Amine	Carbolic coefficient Colorless	Colored
Aniline	0.57	0.69
o-Toluidine	1.00	1.20

The effect of exposure is therefore to increase slightly the germicidal powers of these amines.

This fact is of interest in relation to the work of Thalhimer and Palmer (*Journal of Infectious diseases*, Vol. IX, 1911, p. 172) who have recently shown that phenol which had become colored either by age or by exposure to sunlight also possesses a higher germicidal power than fresh colorless phenol.

The authors desire to express their thanks to Dr. C. J. Martin, F.R.S., for laboratory facilities given at the Lister Institute and to the Government Grant Committee of the Royal Society for a Grant which has partly defrayed the cost of the chemicals employed in this investigation.

ON THE LENGTH OF LIFE OF THE RAT-FLEA APART FROM ITS HOST.

BY

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It is unnecessary to make any lengthy reference to the important part which fleas may play in spreading disease, not only amongst men, but also amongst animals. They have been shown to be intimately connected with bacterial, protozoal, and even with helminthic infections, and for this reason it appears desirable that we should have as complete knowledge as possible in regard to their life-history, and especially in regard to such points as may be of importance in their relation to the spread of disease.

Under ordinary circumstances, when in more or less close association with its host, the rat-flea (*Ceratophyllus fasciatus*) passes though its developmental cycle in from two to three weeks. The rapidity of development is considerably affected by the environmental conditions, of which the chief appear to be the temperature and amount of moisture. In warm, damp weather the developmental period may be as short as ten days. Under unsuitable conditions it may be greatly prolonged.

With regard to the actual length of life of adult fleas, the Plague Commission in India found that the Indian rat flea (*Xenopsylla cheopis*) might remain alive for at least forty-one days when fed on rat's blood, but only twenty-seven and twenty days when fed on human and guinea-pig's blood respectively. Without food the fleas did not live longer than fourteen days when moisture was present, and only six days in the absence of moisture.

Gauthier and Raybaud record some experiments in which they kept a rat-flea (*Ceratophyllus*) alive on human blood alone for ninety days. They also found that at low temperatures fleas might live for a considerable length of time without feeding at all. In one experiment a flea remained alive in the ice-chamber for forty-one days without feeding.

The experiments to be described were directed chiefly towards determining how long adult fleas might remain

Table I.—*Showing Effects of Light and Darkness.*

	Date.	No. of Fleas.	Average Length of Life. Days.	Longest.	
1	Nov. 7th, 1910 ...	12	—	40	Light.
2	Jan. 17th, 1911 ...	12	7.0	25	Light.
3	Jan 24th, 1911 ...	12	6.9	16	Dark.
4	Feb. 1st, 1911 ...	6	11.0	22	Light.
5	Feb. 18th, 1911 ...	6	4.8	13	Light.
6	,, ,,	6	4.3	8	
7	June 6th, 1911 ...	6	3.3	5	Dark.
8	,, ,,	6	3.8	4	Shade
9	,, ,,	6	6.1	8	Shade.
10	,, ,,	6	6.3	11	
11	June 12th, 1911 ...	9	2.4	4	Light. No sand.
12	,, ,,	9	3.8	10	Dark. No sand.
13	,, ,,	8	2.7	5	Shade. No sand.
14	June 15th, 1911 ...	6	3.8	7	Shade. Dry sawdust and bread.
15	June 16th, 1911 ...	6	7.1	19	Shade. Sawdust, bread, faeces.
16	June 17th, 1911 ...	8	4.7	10	Shade. Sawdust.
17	Oct. 4th, 1911 ...	6	7.8	12	Light.
18	,, ,,	6	9.3	13	Light.
19	,, ,,	6	6.3	9	Dark.
20	,, ,,	7	9.1	17	Dark.
21	,, ,,	7	5.1	10	Shade.
22	,, ,,	7	6.4	7	Shade.
23	Oct. 18th, 1911 ...	8	5.6	18	Light.
24	,, ,,	8	8.1	21	Light.
25	,, ,,	8	7.5	15	Dark.
26	,, ,,	8	3.5	8	Dark.
27	,, ,,	8	4.3	8	Shade.
28	Oct. 18th, 1911 ...	8	4.8	8	Shade.
29	Nov. 16th, 1911 ...	7	14.8	20	Light. Moisture added.
30	,, ,,	7	15.5	23	Light. Moisture added.
31	,, ,,	7	11.2	24	Dark. Moisture added.
32	,, ,,	7	16.2	24	Dark. Moisture added.
33	,, ,,	7	10.0	18	Shade. Moisture added.
34	,, ,,	7	12.4	19	Shade. Moisture added.
35	Dec. 11th, 1911 ...	7	6.1	9	Light.

TABLE I (*continued*).

	Date.	No. of Fleas.	Average Length of Life.	Longest.	
			Days.		
36	Dec. 11th, 1911 ...	7	6.5	8	Dark.
37	,, ,,	7	6.0	12	Shade.
38	,, ,,	7	10.2	19	Light.
39	,, ,,	7	8.4	14	Dark.
40	,, ,,	7	7.5	11	Shade.
41	Jan. 8th, 1912 ...	7	8.7	13	Light. Slightly damp.
42	Jan. 8th, 1912 ...	7	9.0	13	Dark. Slightly damp.
43	,, ,,	7	5.0	14	Shade. Slightly damp.
44	,, ,,	7	5.4	9	Light. Very damp.
45	,, ,,	7	3.8	7	Dark. Very damp.
46	,, ,,	7	4.2	7	Shade. Very damp.
47	Jan. 18th, 1912 ...	7	5.4	12	Light.
48	,, ,,	7	5.8	14	Dark.
49	,, ,,	7	4.5	11	Shade.
50	,, ,,	7	3.4	8	Light.
51	,, ,,	7	5.4	8	Dark.
52	,, ,,	7	2.4	5	Shade.
53	Jan. 31st, 1912 ...	7	9.2	20	Light.
54	,, ,,	7	3.0	11	Dark.
55	,, ,,	7	5.5	11	Shade.
56	,, ,,	7	3.2	12	Light.
57	,, ,,	7	1.5	6	Dark.
58	,, ,,	7	2.2	11	Shade.
59	Feb. 14th, 1912 ...	7	4.0	5	Light.
60	,, ,,	7	3.8	7	Dark.
61	,, ,,	7	3.1	6	Shade.
62	,, ,,	7	7.1	14	Light.
63	,, ,,	7	6.5	11	Dark.
64	,, ,,	7	6.4	11	Shade.
	Total	463	6.3	40	

alive without food under various conditions. Some experiments, however, were also made with larvae, in order to decide to what extent exactly their life might be prolonged under adverse conditions. The former set of experiments was conducted as follows: Fleas were removed either directly from a rat or from the material in the rat-cage They were then transferred to small, clean, dry flasks, in the bottom of which was placed a small quantity of sterilized sand. The mouth of the flask was loosely plugged with wool. In certain cases the wool plug was slightly moistened from day to day, and in others a trace of moisture was added to the sand at the beginning of the experiment. The conditions of light and temperature were also varied. The flasks were carefully examined at least once every day, and a count made of the fleas still alive.

As a rule, 7 fleas were used in each experiment, and altogether 89 experiments, involving a total of 638 fleas were performed. In the first experiment the number of fleas living from day to day was not noted, but in all the others an accurate record was kept. In the majority of cases the flasks were kept at ordinary room temperature, which, during the daytime in summer, varied from 15° to 23° C., and in winter from 10° to 17° C.

In addition, the following experiments were undertaken to ascertain the effect of keeping fleas continuously at low temperatures. Control experiments were made at room temperature with the same batch of fleas. In one set moisture was supplied by introducing each day a pellet of fresh rat's faeces:

TABLE II.—*Showing Effect of Low Temperatures.*

	Date.	No. of Fleas.	Average Length of Life.	Longest.	
			Days.		
65	June 13th, 1912 ...	7	6.0	10	Temp. 18° C. Light. Without faeces.
66	,, ,,	7	7.7	10	Temp. 18° C. Shade. Without faeces.
67	,, ,,	7	36.4	59	Temp. −1° C. Without faeces.
68	,, ,,	7	39.7	52	Temp. 8° C. Without faeces.
69	,, ,,	7	8.2	21	Temp. 18° C. Light. With faeces.
70	,, ,,	7	9.7	17	Temp. 18° C. Shade. With faeces.
71	,, ,,	7	22.7	74	Temp. −1° C. With faeces.
72	,, ,,	7	43.1	67	Temp. 8° C. With faeces.

In a third series the fleas were maintained continuously at a high temperature (37°), and at an intermediate temperature (24°). (See Table III on page 5.)

From these figures several interesting conclusions may be drawn:

1. The average length of life of *Ceratophyllus fasciatus*, apart from its host under general circumstances, is just

under seven days, but about 9 per cent. live for a fortnight and at least 2 per cent. for three weeks or over (of 505 fleas 46 lived at least fourteen days and 10 at least twenty-one days).

TABLE III.—*Showing Effect of High Temperatures.*

	Date.	No. of Fleas.	Average Length of Life. Days.	Longest.	
73	Aug. 26th, 1911 ...	7	5.0	9	Temp. 24°.
74	,, ,,	7	4.7	9	Temp. 24°.
75	,, ,,	7	4.0	7	Temp. 24°.
76	,, ,,	7	4.1	6	Temp. 18°.
77	,, ,,	7	9.1	16	Temp. 18°.
78	,, ,,	7	Few hours	less than 24 hours	Temp. 37°.
79	,, ,,	7	,,	,,	Temp. 37°.
80	,, ,,	7	,,	,,	Temp. 37°.
81	Aug. 27th, 1911 ...	7	,,	,,	Temp. 37°.
82	,, ,,	7	,,	,,	Temp. 37°.
83	,, ,,	7	,,	,,	Temp. 37°.
84	Aug. 28th, 1911 ...	7	,,	,,	Temp. 37°.
85	,, ,,	7	,,	,,	Temp. 37°.
86	,, ,,	7	,,	,,	Temp. 37°.
87	Sept. 2nd, 1911 ...	7	,,	,,	Temp. 37°.
88	,, ,,	7	,,	,,	Temp. 37°.
89	,, ,,	7	,,	,,	Temp. 37°.

2. Other things being equal, they live longer in winter than in summer, that is, longer at low temperatures than at high. Under ordinary circumstances, when the temperature is over 15° C. for any considerable part of the time, it would be exceptional for them to live, without feeding, for more than forty days, but, from experiments 65 to 72, it is evident that in winter, when the temperature remains continuously under 10° C. (50° F.), they may remain alive for as long as two months, and if the temperature is maintained continuously at freezing point this period may be extended to over ten weeks.

3. Above 25° C. the length of life is greatly curtailed. and at 37° C. it is always less than twenty-four hours, though in some cases more than twelve hours.

4. Both excess of dryness and excess of moisture curtail the length of life.

5. Conditions of light do not appear to have any great influence, but in these experiments the fleas exposed to bright daylight lived on an average slightly longer than

those kept in darkness or in the shade, the average figures being respectively 6.9, 6.6, and 5.8 days.

6. In the experiments which were done to determine whether the fleas could live longer when removed directly from the rat or from the material in the rat-cage, it was found that in the latter case the average length of life was two and a-half days longer, the average figures being 4.1 and 6.8 days respectively.

With regard to the length of life of larval fleas, some observations I was able to make may be of interest:

1. On September 14th, 1910, a rat in one of the flea cages died and the contents of the cage, consisting of grain, sawdust, faeces, etc., and containing a large number of fleas and larvae, were removed and put into a tall glass vessel, which was securely covered and placed in a cupboard. On examining this vessel two months later it was found that the number of fleas did not appear to have materially decreased. On December 19th quite a number of active fleas were present, and on January 29th, 1911, a few still remained. Observations were continued throughout February, and on February 17th a few fleas were observed, but this was the last date on which any were seen, although periodical observations were made throughout March. April, and May.

2. A similar experiment was started on December 5th, 1910. Observations were made once a week and it was found that the number of fleas apparently fell off towards the end of January, 1911, but increased again during February. Thereafter, however, they steadily decreased until May 24th, when none were visible. The observations were continued, and in October a few fleas again appeared. They were seen up till the middle of November, after which no more appeared, although the material was kept until January 30th, 1912.

3. A third experiment of the same nature was started on February 10th, 1911. The fleas were observed during the ensuing months. On November 7th a considerable number were present. Some were removed and placed along with two young rats free from fleas. The fleas settled on the rats, but not with such avidity as was expected. One rat died within twenty-four hours, the other the following day. Bacteriological examination of the blood and intestine of the rats did not afford any clue as to the cause of death. On November 28th only one flea was seen. It was removed and died shortly afterwards. In December, however, a number of fleas made their appearance, and on January 30th several fleas and larvae were observed. Next day five fleas were removed and placed in a large jar with a young rat free from fleas. The fleas attacked the rat almost immediately. Five days later the rat died. Bacteriological examination again was negative, though, it must be remarked, no anaërobic cultures were made. There were no marked *post-mortem* appearances. In this case a control rat from the same brood was used and placed under the same conditions as the first, but without fleas. It remained well. On February 5th, when the first rat died, the second was placed in the jar from which the first was removed. It remained alive and well for three months. During the first month no increase in the number of fleas could be detected, but thereafter the number gradually increased until, at the end of another six weeks, the jar contained a very large number of fleas.

The original material in the above experiment was kept until April 3rd, when, on repeated examination, no fleas or larvae could be observed.

In all the foregoing experiments the material used was comparatively dry, and in the course of time it became as dry as dust in the upper layers, although there was usually an appreciable degree of moisture in the bottom of the vessel. In two other experiments, however, the original material was obviously damp. In the first of these the fleas had disappeared entirely at the end of two months. In another month the material was covered with white mould and no fleas or larvae were seen then or at any later period. The second experiment had practically the same result.

In a third set of these experiments 30 fleas were removed from the rat cage and placed in a large vessel containing some comparatively dry, clean bran. In each case in the course of a month the fleas had entirely disappeared and no larvae or pupae could be detected in the bran. The same result was obtained when fleas from Experiments 2 and 3 of the first set were used. This appears to show that under these circumstances the fleas did not breed.

From these experiments the following conclusions may be deduced ;—

1. Material infected with fleas and larvae may remain infected for as long as a year.

2. This appears to be due to the fact that the larval and pupal stages in the development are greatly prolonged under the adverse conditions. Dampness, however, rapidly kills off both fleas and larvae.

3. No direct evidence was obtained that the fleas bred under these conditions; but the appearance of larvae towards the end of some of the experiments suggests that they did breed to some extent.

4. Fleas derived from the infected material readily attacked rats, and proceeded to multiply in the course of a short time.

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ON TWO NEW TREMATODE PARASITES FROM BRITISH FOOD-FISHES.

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(With Plate IX.)

In September, 1908, an opportunity was afforded me of examining some of the larger food-fishes brought in to Aberdeen fish market. For this I have to thank the courtesy of Professor J. Arthur Thomson, who kindly provided the necessary laboratory accommodation, and was of great assistance to me in various other directions. I have also to thank the Government Grant Committee of the Royal Society for defraying the expenses of procuring specimens.

I was enabled to obtain several species of fish which I had not met with during my earlier investigations at St Andrews. Amongst these were a few specimens of the horse-mackerel (*Trachurus trachurus*) and the sea-bream (*Sparus centrodontus*), each of which furnished a new and interesting species of parasite. During the short time at my disposal only a couple of horse-mackerel and a dozen bream were obtained and the number of Trematode parasites they contained was very small. A larger number of these fishes have since been collected in various localities, chiefly at Plymouth, but they have added only sparingly to the number of parasites. The form in the horse-mackerel has indeed not been met with again; that from the bream has been found on seven other occasions. Both forms present several interesting peculiarities, and they are not only new species but are types of new genera. For the parasite from the horse-mackerel I propose the name, *Ancylocoelium typicum*, n.g., n.sp., and for that from the bream, *Zoonogenus vividus*, n.g., n.sp.

Ancylocoelium typicum, n.g., n.sp. (Pl. IX, fig. 1).

This is a moderately small Trematode measuring 2·25 mm. in length and 0·48 mm. in maximum breadth. It is decidedly flattened dorso-ventrally and its outline is somewhat spatulate, the greatest breadth occurring in front of the middle of the body, whence it tapers sharply to the anterior end and more gradually to the posterior end. The average breadth in the posterior half of the body is 0·28 mm. In life the animal has a rich yellow colour from the enormous number of eggs which it contains. The cuticle is beset for the greater part of its extent with small scaly spines, which disappear gradually towards the posterior end.

The suckers are small and inconspicuous. The subterminal oral sucker has a diameter of 0·10 mm., while the ventral sucker measures ·13 mm. The latter is situated at a distance of 0·92 mm. from the anterior end.

The somewhat elongated pharynx follows almost immediately on the oral sucker and measures 0·08 × 0·043 mm. The oesophagus is long and narrow. Its length is about 0·5 mm. and the bifurcation takes place a short distance in front of the ventral sucker. The intestinal diverticula are of unique configuration. They are considerably wider than the oesophagus and instead of passing round the ventral sucker they are bent back abruptly into the anterior part of the body. They diverge at a fairly wide angle, passing out towards the sides of the body, but when they have nearly reached the margin they are again bent somewhat acutely and pass back almost straight. They terminate a short distance behind the level of the intestinal bifurcation.

Owing to the enormous mass of eggs, only the terminal part of the excretory vesicle could be observed even in the living specimen.

The genital system presents several peculiar features. The anterior testis is situated somewhat to the right side, a short distance behind the ventral sucker, from which it is separated by the ovary. The posterior testis lies more median, not far behind the anterior testis (0·22 mm. behind the ventral sucker). Both are elongated oval and measure about 0·19 × 0·10 mm., their long axes being slightly oblique to the long axis of the body. The cirrus-pouch is of simple type and is situated over and in front of the right anterior quadrant of the ventral sucker. Posteriorly it is almost contiguous with the ovary and the anterior testis. It shows the usual shape and contains a large oval vesicula seminalis. Its dimensions are 0·32 × 0·11 mm. There is a very short pars prostatica and a comparatively short and straight ductus ejaculatorius, no part of which was exserted. The genital aperture is situated in the middle line between the intestinal bifurcation and the anterior border of the ventral sucker, but decidedly nearer the former.

The ovary is wedged in between the anterior testis, the ventral sucker and the cirrus-pouch. It is about the same size as the sucker (greatest diameter 0·14 mm.) and its outline is almost globular. The presence of a receptaculum seminis or Laurer's canal could not be ascertained owing to the enormous number of eggs in the vicinity of the ovary. The yolk-glands are situated in the sides of the body at the level of the testis. They are slightly asymmetrical, that on the left being a little in advance of the other. The latter extends from the anterior border of the anterior testis to about 0·7 mm. from the posterior end of the body. They appear to have an unusual configuration, the follicles being arranged in tubules or rather in one continuous tubule which is thrown into a rosette-shaped knot; the rays of the rosette, however, are not equal, those directed forward being decidedly longer than those directed backward. On ventral view only the lateral aspect of the rosette is seen. The yolk-ducts were not observed.

The uterus, which fills the remainder of the posterior part of the body, is extremely voluminous and contains a very large number of small eggs. The uterine walls could not be made out distinctly, but it seems certain that the uterus is not convoluted, the enormous number of eggs being accommodated by a dilatation of the uterine tube. This runs from the ovary, on the right side, backwards to the end of the body, where it turns and passes forward on the left, overlapping the descending limb to some extent. It still retains a considerable width even on passing in front of the ventral sucker and it is joined to the small genital sinus by a very short vagina.

From the genital sinus another unusual and peculiar structure arises. This is a pear-shaped pouch with a narrow neck, which lies between the left intestinal diverticulum and the uterus. The latter separates it from the ventral sucker. It is smaller than the cirrus-pouch, about 0·24 × 0·11 mm., and appears to contain no structure or contents. It seems to be simply a thin-walled sac, and no clue is afforded to its function.

The mature eggs are bright yellow in colour, with oval outline and measure on an average 0·024 × 0·013 mm.

This form possesses several remarkable features which distinctly mark it off as a new type. The chief of these are the accessory genital sac, the unique configuration of the intestinal diverticula and the peculiar condition of the yolk-glands. It displays no close affinity to any hitherto described Trematode but for systematic purposes it may, for the present, be placed in the vicinity of the *Haplocladinae* (Odhner 1911).

Zoonogenus vividus, n.g., n.sp. (Pl. IX, fig. 2).

This is neither such a remarkable, nor such an uncommon, species as the preceding. It was met with in two out of 11 specimens of the sea-bream (*Sparus centrodontus*) examined at Aberdeen (September) and I have since met with it in five out of eight bream examined at Plymouth (July—August). It was absent, however, in half-a-dozen bream obtained from Billingsgate (November). Its frequency, from these figures, is therefore seven out of 25.

The sea-bream does not occur in inshore waters; it is only obtained at a considerable distance from the shore. For that reason all the specimens examined had been dead for some time, and the parasites were usually in a more or less macerated condition. The species, however, is a particularly delicate one, judging from the fact that in the same fishes such forms as *Derogenes varicus* and *Hemiurus communis* were still alive and active.

This new species occurred invariably in the rectum and in no other part of the intestine. Only two or three specimens were found in each case. The worms were easily picked out by reason of their remarkably vivid, blood-red colour. The rectal contents of the sea-bream, partly from the fact that it feeds largely on Crustacea, are usually of a dull brownish red colour, but the colour of the parasite was much more intensely red.

It is a small worm, obviously belonging to the family Zoogonidae, and bearing much resemblance in structure and habitat to the commoner and more familiar species, *Zoogonoides viviparus*. Its length is on an average 1·4 mm. and its breadth 0·46 mm. It is somewhat oval in outline, not much flattened, and the greatest breadth is rather behind the middle of the body. As already remarked, the colour is uniformly blood-red, but on close examination the coloration is found to be blotchy, much as in *Zoogonoides viviparus*. The cuticle is covered in the anterior half with minute regularly arranged spines, but these disappear towards the posterior end of the body.

The oral sucker is subterminal and measures about 0·16 mm. in diameter. The ventral sucker is much larger, 0·34 mm. in diameter, and is situated behind the middle of the body (0·9 mm. from the anterior end). The diameter of the ventral sucker is therefore distinctly more than twice that of the oral sucker and in some cases it is nearly three times. Both suckers are globular.

The pharynx is contiguous with the oral sucker and measures 0·07 mm. in diameter. It is succeeded by a short oesophagus, rather shorter than the pharynx. The intestinal diverticula are simple, narrow and short, terminating some distance in front of the ventral sucker.

The excretory vesicle is a short bulbous sac at the posterior end of the body, containing in many cases a hard yellowish green or brown concretion of irregular shape.

The testes are two ovoid bodies situated one over each posterior quadrant of the ventral sucker, their posterior borders being just a little behind the posterior margin of the sucker. Their long axes are oblique and almost tangent to the edge of the sucker. They measure about 0·25 mm. × 0·12 mm. The cirrus-pouch is a conspicuous arcuate structure lying immediately in front of the ventral sucker. In most cases it is entirely in front of the sucker, but in some it touches or even slightly overlaps it. It contains a bi-partite vesicula seminalis, the posterior half being the larger and measuring 0·1 × 0·07 mm., while the anterior half is almost globular and has a diameter of ·065 mm. In front of this is a bulbous pars prostatica which is slightly larger than the anterior part of the vesicula (0·08 mm.). The rest of the cirrus-pouch (·3 mm.) is traversed by a long ductus ejaculatorius, the walls of which are characteristically crenated or folded. No spines are present in the ductus or cirrus. The genital aperture is situated over the left intestinal diverticulum near its termination. It is therefore not absolutely at the lateral margin of the body.

The ovary is situated in the middle line, at the posterior border of the ventral sucker, between the testes. Sometimes it is a little in front of, sometimes a little behind the testes. It is globular and has a diameter of 0·09 mm. Alongside the ovary, sometimes dorsal to it, is a very small receptaculum seminis, from which passes a short Laurer's canal. The yolk-glands consist of a small compact mass lying near the ovary, sometimes on its right side, sometimes behind it. The uterus lies almost entirely behind the ventral sucker, although one convolution is thrown up along the right side of the sucker. On the left side and in front of the sucker the uterus runs into a highly developed and very muscular vagina. This lies behind the cirrus-pouch and is considerably inflated. The eggs, as in all the members of the family, develop rapidly and a completely formed and actively motile larva is present long before the eggs are laid. The egg-capsules vary greatly in size, those in the initial part of the uterus measuring about 0·016 × 0·012 mm., while in the terminal part they measure 0·036 × 0·018 mm. The egg-capsules are, thus, less than half the size of those in *Zoogonoides viviparus.*

Of the systematic position of this form there can be little doubt. It is without question a member of the family Zoogonidae, and of the subfamily Zoogoninae, bearing a close resemblance to the genera *Zoogonus* and *Zoogonoides.* So closely, indeed, does it resemble the latter, that I was at first inclined to include it in this genus. Odhner's (1911) revision of the family, however, distinctly indicates that it is an intermediate form between *Zoogonus* and *Zoogonoides.* I have on that account made it the type of a new genus. Its generic characters may be summed up as follows: resembling *Zoogonoides* but differing in having the cirrus unarmed, in having a very highly developed vagina, and in possessing much smaller egg-capsules.

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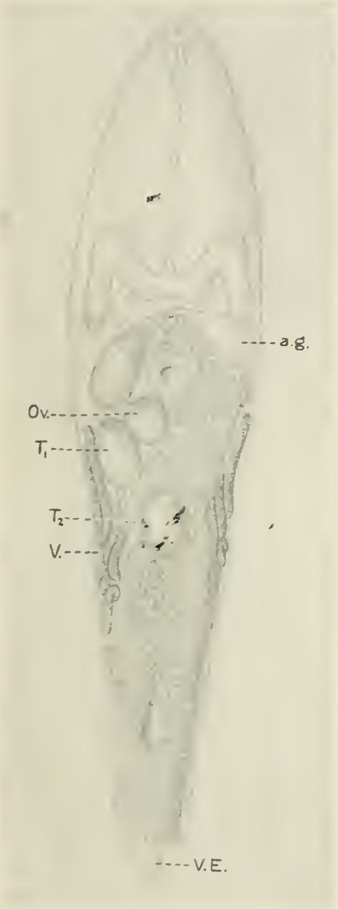

Fig. 1.

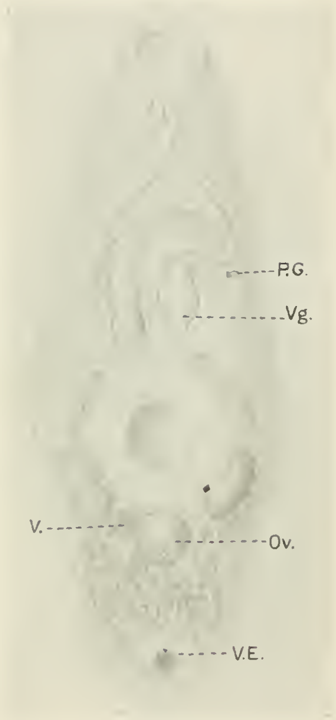

Fig. 2.

M. Rhodes, del.

Fig. 1. *Ancylocoelium typicum.* Ventral view (× 50). a.g., accessory genital sac; Ov., ovary; T_1, T_2, testes; V., yolk-glands; V. E., excretory vesicle.

Fig. 2. *Zoonogenus vividus.* Ventral view (× 65). Ov., ovary; P. G., genital aperture; V., yolk-gland; V. E., excretory vesicle; Vg., vagina.

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On two new Larval Trematodes from the Striped Snake (*Tropidonotus ordinatus sirtalis*).

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(Text-figure 107.)

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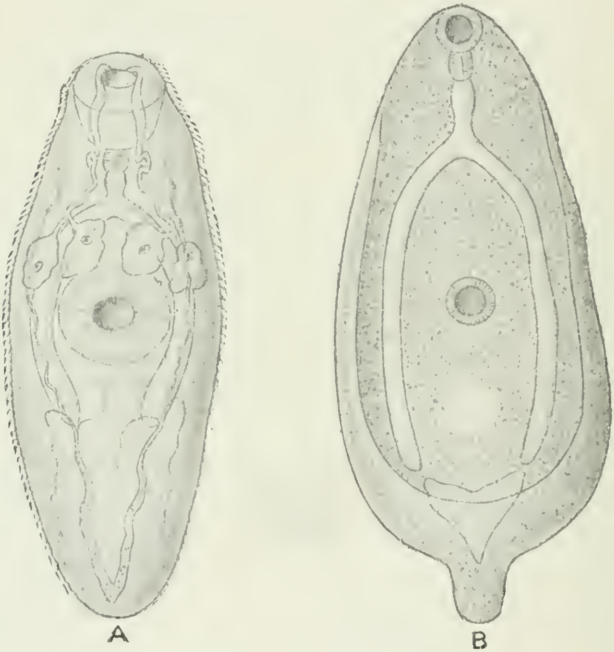
The occurrence of encysted larval parasites in snakes is evidence, if such were wanting, that some snakes are eaten by other animals. What is more important, the character of the parasites may indicate what variety of animal is in the habit of eating the snake in question. Conversely, the presence of any particular species of adult parasite in an animal is almost always a sure proof that such animal eats the snake in which the larval stage is found. It is unfortunately in many cases a matter of difficulty to diagnose the systematic characters of a larval parasite. In a number of cases, however, it is possible to assign it to a definite genus, rarely to a particular species.

The two cases to be dealt with here present a certain amount of difficulty. The first larva is evidently a Distomate Trematode, but beyond that it is impossible to go; the second larva is just as obviously a Holostomid, and almost certainly belongs to the genus *Hemistomum*. Such a diagnosis does not lead very far, but it at least enables one to say that in all probability the Striped Snake (*Tropidonotus ordinatus*, var. *sirtalis*) is eaten by some bird, for adult Holostomata are known to occur only in birds. This will possibly be confirmed by direct observation.

Both forms were met with together in each of three Striped Snakes from North America, which died in the Society's Gardens on the 5th and 20th December, 1910, and on 10th March, 1911. They occurred in enormous numbers in the mesenteric fat along the whole length of the intestine. Each was enclosed in a small spherical or ovoid cyst with unusually thin and soft walls. Unlike what is generally found in a Trematode cyst, the wall gave the impression of being a thin membrane instead of the more usual tough chitinous investment. On this account, not only could the larvæ be extracted from the cysts without difficulty, but when placed in water they escaped readily of their own accord. When a piece of the cyst-infested mesentery was suspended in water, a continuous shower of larvæ was observed to fall to the bottom of the vessel.

The first form, which I name **Cercaria ordinata**, sp. n. (text-fig. 107 A), was much more numerous than the other. It is a typical tailless encysted cercaria, about ·5 mm. (·4–·55 mm.) in length and ·2–·25 mm. in greatest breadth. In shape it is ovoid and flattened dorso-ventrally. The entire surface of the body is covered with minute regularly-arranged spines. The oral sucker is almost terminal and has a diameter of ·07 mm. (·06–·08 mm.). The length is usually slightly greater than the breadth, and the sucker has a somewhat characteristic funnel-shaped appearance. The globular ventral sucker is situated rather in front of the centre of the body, and has a diameter of ·083 mm. (·075–·097mm.). Its distance from the anterior end of the body is on an average ·22 mm. (·17–·25 mm.). On the dorsal lip of the oral sucker are the two symmetrical apertures of the cystogenous ducts. The cystogenous glands are conspicuous structures, and consist of four

Text-fig. 107.



A. *Cercaria ordinata*, sp. n. Ventral view, × 150.

B. *Diplostomum sirtale*, sp. n. Ventral view, × 150.

large cells situated in a transverse row immediately in front of the ventral sucker. There are two pairs, a right and a left. The ducts from each pair unite almost at once, and the united ducts then pass forward in an irregular course; but just before they reach the oral sucker each makes a characteristic twist, following which there is a gradual increase in calibre until near the termination, when they contract slightly again. The cystogenous glands have an irregular rectangular outline, and measure ·048 × ·037 mm. In direct contact with the oral sucker is the small muscular pharynx measuring ·024 mm., which is continued by a short, somewhat dilated œsophagus of the same length as the pharynx. The intestinal bifurcation is about midway between the two suckers. The diverticula are simple, somewhat wide tubes, which terminate not far behind the ventral sucker (*i. e.* a third of the distance from the sucker to the posterior end of the body). The excretory vesicle is V-shaped. A common trunk is practically absent, and the limbs extend forward to near the terminations of the intestinal diverticula. The excretory tubules are very fine; the main tube on each side extends forward to near the oral sucker, where it turns back. The excretory aperture appears to be slightly dorsal.

No trace of other organs could be made out, so that no accurate idea can be obtained of the systematic position of this larva. The peculiar configuration of the excretory vesicle may, in conjunction with the shape of the alimentary canal, eventually lead to the identification of its adult form, but at present, so far as I am aware, there is no known adult Distome to which this larva can be ascribed.

The second form, which I name DIPLOSTOMUM SIRTALE, sp. n. (text-fig. 107 B), differs markedly from the first. It is about the same size, and occurs in somewhat similar cysts, but its shape and colour are entirely different. The body of *Cercaria ordinata* is light and transparent, whereas that of *Diplostomum sirtale* is dark and almost opaque. The opacity is due to the presence of innumerable small granules distributed throughout the whole body. The shape is that of a typical *Diplostomum* larva, being scoop-like with a short handle. The shape is due to the rolling over of the postero-lateral margins of the body. In life, however, these margins are capable of more or less eversion, so that on occasion the body may appear almost flat. The dimensions of this larva are ·48–·55 × ·28–·32 mm., the short stumpy tail being ·06 mm. long.

The oral sucker measures ·039 mm. in diameter; the ventral sucker ·042–·045 mm. The latter is situated a little in front of the middle of the body, ·25–·28 mm. from the anterior end. Midway between it and the posterior end of the body occurs the characteristic Holostomid fixing disc, which appears as a transparent disc about the same size as the ventral sucker. Of the internal organs only the alimentary canal and excretory vesicle were visible. The former comprises a pharynx contiguous with the oral sucker and measuring ·024 × ·020 mm. This is followed by an œsophagus about twice as long as the pharynx. The intestinal bifurcation takes place rather nearer the ventral sucker than the oral sucker, and the simple diverticula extend a little beyond the posterior border of the fixing disc. The excretory vesicle consists of a wedge-shaped sac, which extends forward as far as the ends of the intestinal diverticula.

This is in all probability the larval stage of some species of *Hemistomum*, parasitic in a bird.

[*From the* Proceedings of the Zoological Society of London, 1912.]
[Published December 1912.]

On Two new Trematode Parasites from the Indian Cobra.

By William Nicoll, M.A., D.Sc., M.D., F.Z.S., Lister Institute of Preventive Medicine, London.

(Text-figure 122.)

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In an Indian Cobra (*Naja tripudians*) which died in the Zoological Society's Gardens on 11th October, 1911, a few specimens of two interesting new Trematode parasites were found. Of the first of these a single specimen was found in the gall-bladder; of the second, four somewhat macerated specimens were met with in the ureters. Four different species of Nematode parasites were also present in the same animal, so that altogether it was infected with six different species of parasitic worms.

The first species belongs to the family Dicrocœliidæ and is of particular interest from the fact that members of this family are not commonly found in Reptiles. Hitherto the only typical representative in Reptiles is that described by de Faria (1910). The present species shows most of the characteristic features of the family, but at the same time it exhibits several divergences of such importance as to warrant its being regarded as the type of a new genus. For that genus I propose the name *Xenopharynx*.

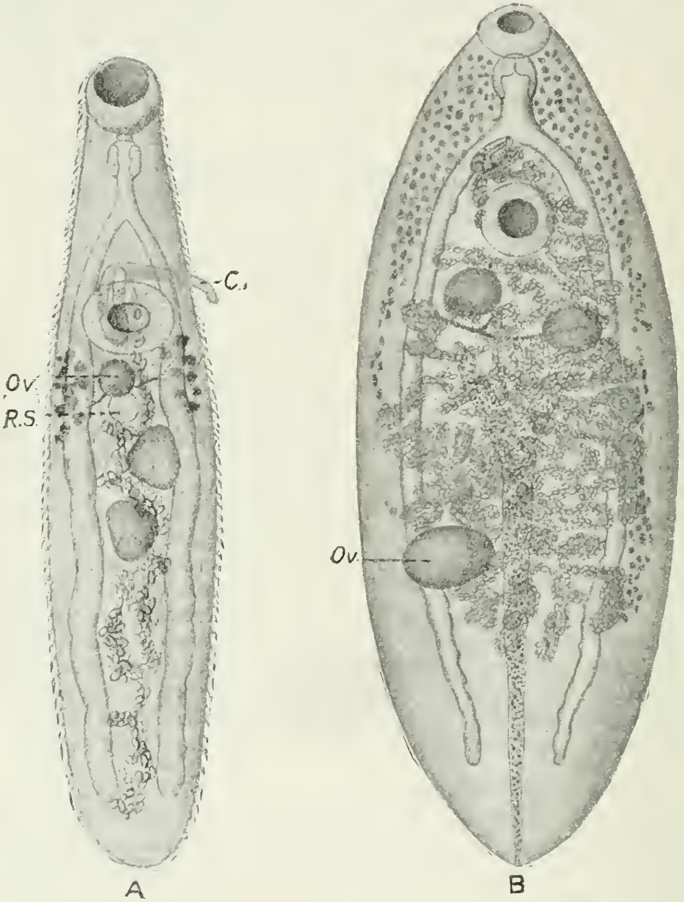
Xenopharynx solus, gen. et sp. n. (Text-fig. 122, B.)

Only a single specimen was found in the gall-bladder. It measures 4·55 mm. in length and 1·68 mm. in greatest breadth, which is about the middle of the body. The outline is almost elliptical and the body is fairly flat. There are no cuticular spines.

The oral sucker has a diameter of ·42 mm., but its length is only ·30 mm. The ventral sucker is circular, with a diameter of ·41 mm. It is situated 1·13 mm. from the anterior end. The neck is therefore almost exactly one-fourth of the body-length.

The pharynx is contiguous with the oral sucker and measures ·22 × ·24 mm. It possesses the curious shape shown in text-fig. 122, B. At first sight this shape was thought to be the result of unequal contraction of the pharyngeal walls or perhaps due to the drawing of the œsophagus into the lumen of the pharynx, but neither of these possibilities seemed to accord with experience. The condition appears to be brought about by the thinning of the posterior part of the pharyngeal wall, which anteriorly is of the usual thickness. In section the appearance is somewhat horseshoe-shaped. It is unfortunate that no second specimen was

Text-fig. 122.



M. Rhodes del.

A. *Styphlodora najæ*, sp. n. Ventral view. × 40. *C.*, cirrus; *Ov.*, ovary; *R.S.*, receptaculum seminis.

B. *Xenopharynx solus*, gen. et sp. n. Ventral view. × 25. *Ov.*, ovary.

available for comparison. Following the pharynx, and about as long as it, is a wide œsophagus, which bifurcates about midway between the pharynx and the ventral sucker. The diverticula are fairly uniform, but become somewhat sinuous towards their termination, which is ·45 mm. from the end of the body.

The excretory vesicle is **Y**-shaped, with a long median stem reaching the middle of the body and two short limbs, the left of which extends as far forward as the left testis. Owing to the fact that sections could not be made, it was impossible to determine whether Odhner's view with regard to the shape of the excretory vesicle in the Dicrocœliidæ holds good in this case. To all appearance, however, the paired limbs are part of the vesicle. Their walls stand out as distinctly, and are about the same thickness, as those of the main stem.

The testes lie not far behind the ventral sucker. They are two transversely oval bodies, separated from each other by a distance equal to their diameter. They are situated obliquely, the right being about half a diameter in advance of the left, and being separated from the ventral sucker by a similar distance. They measure ·31 mm. in transverse diameter. The cirrus-pouch is of small size and lies entirely in front of the ventral sucker. It measures ·29 × ·13 mm., and contains a small convoluted vesicula seminalis, a comparatively long pars prostatica, and a short narrow ductus ejaculatorius. The genital aperture is median, just over the intestinal bifurcation.

The ovary lies a considerable distance behind the testes, about midway between them and the end of the body. It is on the right side overlying the right intestinal diverticulum. Its outline is transversely oval and it is much larger than the testes, measuring ·31 × ·47 mm. There appears to be no receptaculum seminis, or if present it is obscured by the uterus. The yolk-glands are situated chiefly in the neck, the sides of which they almost completely fill. They extend on each side from the oral sucker to well behind the ventral sucker, but they are more extensive on the left than on the right. On the right they stop at the level of the bifurcation of the excretory vesicle, although there are a couple of small follicles near the ovary. On the left they extend slightly beyond the level of the ovary. In the neck the follicles spread well in towards the mid-line of the body, but behind the ventral sucker they lie entirely to the outer side of the intestinal diverticula. The transverse yolk-ducts cross the body about the level of the testes, and the shell-gland lies just behind the right testis.

The uterus is moderately voluminous and is thrown into irregular narrow convolutions. It does not extend much behind the ovary, but in front it overlaps the intestinal diverticula, and winding between the testes it passes forward on the left side of the ventral sucker and makes a few short turns in front of the sucker before passing into the short vagina. The eggs are small and numerous, measuring ·036–·039 × ·018–·019 mm.

The new species differs from all other Dicrocœliidæ in the distribution of the yolk-glands and in the shape of the pharynx. Another important feature is the distance separating the ovary from the shell-gland complex. From the genus *Dicrocœlium* it is further distinguished by the position of the testes and the ovary and by the extent of the uterus. With *Platynosomum* it is more closely allied, yet the differences between it and this genus are greater than those separating *Dicrocœlium* from *Platynosomum* or *Eurytrema*. It is rather curious that this new species does not show any particularly close relationship to "*Dicrocœlium*" *infidum* de Faria from the snake, *Eunectes marina*. The latter is closely allied to the avian genus *Platynosomum* and should be included in it, unless de Faria's doubtful observation in regard to the excretory vesicle proves to be correct.

STYPHLODORA NAJÆ, sp. n. (Text-fig. 122, A.)

Four somewhat macerated specimens of this species were found in the ureters of the Cobra. The species is a typical member of the genus *Styphlodora* and presents a very great resemblance to *S. serrata* Looss and to *S. horrida* Leidy. The features separating it from these two species are so slight that I have some hesitation in regarding it as a distinct species. They are, however, quite as distinctive as those separating the above two species from each other. The difficulty is rather increased by the fact that the present specimens, although mature, are possibly not fully grown.

The length is 2·0–2·4 mm., the greatest breadth ·42–·55 mm., which occurs a little behind the ventral sucker. The breadth of the whole postacetabular region is fairly uniform, and there is only a very slight attenuation in the neck. There is a considerable amount of dorso-ventral flattening. In each of the specimens cuticular spines were entirely absent, but it is practically certain that they have fallen off, and it would be unreasonable to suppose that this is an unarmed form. On that account I have had spines depicted in the drawing.

The oral sucker is subterminal and has a diameter of ·22–·25 mm. It is rounded and rather shallow. The ventral sucker is slightly transverse and measures ·24 × ·25 mm. It is only very little larger than the oral sucker, and it is situated at a distance of ·67–·84 mm. from the anterior end, *i. e.* about one-third of the body-length. There is a distinct prepharynx followed by a large pharynx measuring about ·13 × ·14 mm. The œsophagus is about the same length, and the bifurcation takes place midway between the suckers. The intestinal diverticula are fairly straight and of considerable width. They are longer than in the other species of *Styphlodora*, reaching to within ·18–·29 mm. of the posterior end. They are very slightly unequal in length.

The main excretory vesicle was entirely invisible, but it was apparent that it gave off numerous lateral branches, which, anastomosing freely, gave the body a honeycomb-like appearance.

The testes lie obliquely behind one another, the left being in front. They are separated by the uterus, which passes between them, but does not overlap them to any great extent. Their outline is irregular, but they appear to be roughly triangular or trilobate. The anterior testis lies almost exactly midway between the two ends of the body, but its position in relation to the ovary varies somewhat, and it may be slightly nearer than is shown in the figure. The posterior testis is about ·27 mm. behind the anterior. They touch the intestinal diverticula or overlap them to a very small extent. Their dimensions are, on an average, ·21 × ·17 mm. and ·22 × ·19 mm. The cirrus-pouch is of moderate length and uniform width, measuring ·31 × ·11 mm. It extends to about the posterior border of the ventral sucker, and contains a convoluted vesicula, a short pars prostatica, and a fairly long ductus ejaculatorius. In each of the specimens the cirrus was exserted and was longer than the pouch itself. The genital aperture is situated in the middle line, almost immediately in front of the ventral sucker.

The small round ovary lies just behind the ventral sucker, a little to the right side, and measures ·13 mm. in diameter. Immediately behind it lies a somewhat smaller receptaculum seminis. The yolk-glands lie on each side at the level of the ovary. They are, for the most part, external to the intestinal diverticula, but they also overlap them dorsally. They consist on each side of about a dozen fairly large follicles, which extend from the posterior border of the ventral sucker to the anterior border of the left testis. The transverse yolk-ducts cross the posterior border of the ovary.

The uterus is poorly developed. It passes back between the testes to near the posterior end of the body and returns along much the same path, terminating in a short vagina. It is only slightly convoluted and contains a comparatively small number of eggs. It is confined within the space bounded by the intestinal diverticula, and it does not form any convolutions behind the ends of the intestine, as is the case in the other species of *Styphlodora*. The ova are rather elongated, oval, possessing a large operculum and, in a few cases, a small knob at the anopercular pole. They measure ·038–·048 mm. × ·019–·024 mm., the average size being ·043 × ·021 mm.

The features which appear to distinguish this form as a definite species are the length of the intestinal diverticula and the restricted extent of the uterus. It is not impossible that both these features may be less marked in a fully-grown specimen. In addition the yolk-glands are slightly less extensive than they are in the other species of the genus. From *S. serrata* it is further distinguished by the relatively larger size of the suckers. Both *S. condita* and *S. horrida* also possess relatively smaller suckers, and in these species the distance between the suckers is considerably less. The only other species of *Styphlodora*, namely *S. similis* Sonsino and *S. bascaniensis* Goldberger, present marked features of difference from the present species.

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[*From the* PROCEEDINGS OF THE ZOOLOGICAL SOCIETY OF LONDON, 1912.]
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Statistical Note on the Worm Parasites collected from the Animals dying in the Zoological Gardens, from December 1910 till April 1912. By WILLIAM NICOLL, M.A., D.Sc., M.D., F.Z.S., Lister Institute of Preventive Medicine, London.

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At the scientific meeting of the Society held on May 21st last, I referred to the excellent work which is being done by the Prosectorial department in acquiring information concerning the parasites which infect the animals living in the Gardens. This is particularly noteworthy in regard to the worm parasites, about which many valuable facts have been obtained. Following my remarks at the meeting, Dr. Beddard suggested that I might be able to supply some general information regarding the animals which had been sent to me for examination, and acting on this suggestion I venture to offer the following communication.

The scheme, which owes its initiation to the Secretary of the

Society, was put into action in November 1910, and the practical working has been evolved as follows: A general examination of the viscera of all the animals killed or dying in the Gardens is made by the Pathologist, after which certain selected examples are forwarded to me for further examination. The desirability of this latter procedure is shown by the fact that a very large proportion of Entozoa are too small to be detected in the course of an ordinary routine examination; and although the detection of such forms does not involve any very special skill, yet it is a tedious process and one which necessitates some experience. In justification of this extra trouble it may be remarked that the comparatively few animals which have been submitted to this additional examination have yielded almost as large a number of parasitic worms as all the other animals put together. This fact, I venture to believe, is a plea for a still more extended and exhaustive system of examination, dealing with all the animals which die in the Gardens. This, naturally, would involve some additional expenditure, which the Society is probably not at present in a position to incur, but it would certainly yield a very rich return.

In addition to supplying information with regard to the diseases and habits of the animals living in the Gardens, as was the original intention of the scheme, it also provides opportunities of studying the parasitic fauna of the various countries from which the animals come. This is a very important consideration, for it is an unfortunate fact that in most of the large faunistic expeditions which have hitherto been undertaken practically no attempt has been made to deal with the internal parasites. As important exceptions to this may be mentioned the German Expedition to Spitzbergen in 1898, and the Swedish Expedition to Egypt and the White Nile in 1901. By both of these expeditions a large number of parasitic forms has been collected, and the results have been published in a series of very valuable monographs. Dr. Leiper has, at these meetings, on more than one occasion called attention to this regrettable neglect of the parasitic fauna, and has urged on collectors the great service they would render by making even a cursory attempt to collect parasitic worms.

During the past seventeen months a total of 198 animals have been submitted to me for examination, the great majority of which have been birds and reptiles. Of these, 87 were found to be infected with worm parasites of one kind or another, *i.e.*, about 44 per cent. In most cases the infection was single, only one species of parasite occurring; but in an Indian Cobra as many as six different species were found. As is generally the case, Nematode infections were much more numerous than any others, there being 60 cases. Trematodes were found in 28 cases, and Cestodes in 15. The Trematodes belong to about 20 distinct species, a large proportion of which are new, and several of which are new generic types.

From the reports of Dr. Beddard and Dr. Leiper I gather that during the same period 41 additional cases of infection with tapeworms, and 53 of infection with Nematodes have been observed. To these must be added a very considerable number of infections with larval Filariæ observed by Mr. Plimmer. These figures, excluding the larval Filariæ, give a total of about 180, of which the infection with Trematodes, Cestodes, and Nematodes is respectively 28, 56, and 113. This appears to show that Trematode parasites are much rarer than other forms, but that is certainly not the case. It must be remembered that only a comparatively small number of the animals passing through the Prosectorium were adequately searched for Trematodes. The extent to which Trematodes and the smaller intestinal Nematodes escape notice except when thoroughly searched for may be gathered from the following table.

	Trematodes.	Cestodes.	Nematodes.
Total number of cases of infection	28	56	113
Number of cases found only on further examination	20	15	60
Percentage	71	26	53

It may be added that the bulk of the Cestoda which were not noted on first examination were small immature forms or fragments of no value. It may therefore be safely affirmed that only a negligible fraction of the mature tapeworms actually escape detection in the Prosectorium. The matter, however, is different with Trematodes; and in view of the fact that only about one-tenth of the total number of animals dying in the Gardens have been submitted to a thorough examination, it must appear that the number of Trematodes actually occurring is considerably greater. Even allowing that they only occur in 5 per cent. of cases, which is taking an extremely low estimate, we ought to have had instead of 28 records a total of well over 100. In the same way the number of small intestinal Nematodes ought to be much increased.

These remarks, however, are not intended as a criticism of the efforts of the prosectorial staff. Parasitic worms are, after all, only a very small part of the work of this department of the Society, and as I have mentioned in the beginning of this communication, considerable praise is due for the results actually obtained. My chief intention is to show that parasitic worms are undoubtedly an important concern and that their importance justifies the attention which is being paid to them. Their claim to notice had until recently been somewhat neglected by the Society, and it seems necessary that someone should offer a plea for their vindication. There can be no doubt that the attention which the Society, thanks to the efforts of Dr. Chalmers Mitchell, is devoting to these forms will do much to benefit Zoology as a whole.

[4]

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NEW TREMATODE PARASITES FROM FISHES OF THE ENGLISH CHANNEL.

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(With Plate XI.)

During August and September 1909 I had an opportunity, thanks to the Government Grant Committee of the Royal Society, of making a fairly comprehensive examination of the parasites of fishes from the English Channel at the Marine Biological Laboratory, Plymouth. During that time nearly five hundred fishes, belonging to about 80 species, were dealt with and a very extensive collection of parasites made. A considerable number of species were obtained many of which are new to British waters but the number of absolutely new species amounted to very few. Of these I am describing here four of the most noteworthy, three of which appear to be of new generic type. A complete account of the investigations will be published later.

Three of these species were rather uncommon. The fourth was comparatively frequent in the pipe fishes and was found in association with a better known species of the same genus, namely *Podocotyle atomon.*

Podocotyle syngnathi n. sp.

(Figs. 1, 2.)

This species was a not uncommon parasite of the pipe fishes, *Syngnathus acus, Nerophis aequoreus* and *Siphonostoma typhle.* In the first it occurred in four out of nine, in the second in four out of eight and in the third in one out of six. It was not met with in any of the eight specimens of *Nerophis lumbriciformis* examined. Its frequency in those fishes is therefore nine out of 31, *i.e.* about 29 %. It did not occur in any other species of fish so that it is in all probability a specific parasite of the Lophobranchii. In *Nerophis aequoreus* it was accompanied by *Podocotyle atomon.*

It bears a very close resemblance to the other species of *Podocotyle* but differs from them chiefly in the noticeable shortness of the cirrus pouch.

The specimens varied in length from 2·2 mm. to 5·9 mm. The smallest specimen was obtained from *Nerophis aequoreus* and had just begun to produce ova, so that evidently this species does not attain maturity so early as *P. atomon.* Some mature specimens of the latter measuring little over 1 mm. were obtained from the same fish.

Measurements were made of five of the largest specimens, the average length of which was 5·36 mm. (4·55–5·95 mm.). The greatest breadth (0·78 mm.) occurred usually at the testicular region, but the breadth across the ventral sucker was almost as great and in one case greater. The greatest breadth is therefore about 1/7 of the length. In a fully extended specimen it may be as small as 1/9. The species is therefore more elongated than *P. atomon* but less so than *P. reflexa.* The body is not much flattened and the ventral sucker is usually prominent.

The oral sucker has a diameter of ·38 mm. and the ventral sucker measures 0·43 × 0·55 mm. The latter is situated at a distance of 1·25 mm. from the anterior end, the neck therefore being somewhat more than 1/4 of the body length.

There is a very short prepharynx and the pharynx measures 0·19 mm. in diameter. The oesophagus is about as long as the pharynx (0·17 mm.), and the intestinal diverticula extend nearly to the posterior end of the body becoming slightly dilated during their course.

The excretory vesicle reaches the anterior border of the ovary.

The testes are separated from each other by a short distance (0·21 mm.). They are oval with their long axes slightly tilted from the middle line, the anterior end in both cases being usually directed towards the left side. They overlap the intestinal diverticula only to a very slight extent. Their dimensions are 0·57 × 0·47 mm. Between the posterior testis and the end of the body there is a space a little over 1 mm. (1/5 of the body length, but this varies from 1/6 to 1/4). The genital aperture is situated a little in front of the intestinal bifurcation, decidedly towards the left side. The cirrus pouch is short and rather stout, extending at most not further than the middle of the ventral sucker. The vesicula seminalis is accordingly much more compact than it is in the other species of the genus. The ductus ejaculatorius is also short.

The ovary lies in front of the anterior testis and is separated from it by a variable space (about 0·1 mm.). It has the trilobate posterior border characteristic of the genus and measures about 0·42 × 0·29 mm. The shell-gland complex resembles that in *P. atomon* but the receptaculum seminis lies rather further forward. The yolk glands are somewhat more limited in extent, stopping a short distance behind the ventral sucker. They are most voluminous posteriorly but are frequently interrupted at the level of the second testis on one or both sides. They do not fill up the inter-testicular space. The transverse yolk ducts unite in front of the ovary. The uterus extends between the receptaculum seminis and the ventral sucker and is confined between the intestinal diverticula. It usually contains about 50 eggs, measuring 0·082–0·102 × 0·045–0·050 mm., the average being 0·092 × 0·047 mm.

Lepidauchen stenostoma n. g., n. sp.

(Fig. 3.)

Only two specimens of this form were found on one occasion in the intestine of *Labrus berggylta*, and it is apparently an uncommon parasite. It is a moderately flat and broad species measuring 2·9–3·25 mm. in length and 1·3 mm. in greatest breadth which occurs about the middle of the body. It tapers slightly towards the ends, which are broadly rounded.

The surface of the anterior part of the body is closely covered with stout spines but these appear to be entirely absent in the posterior part beyond the middle of the body.

The globular oral sucker is subterminal and has a diameter of 0·55 mm. It is characterised by a peculiar aperture which presents the appearance of a longitudinal slit inflated at its anterior end. The ventral sucker is much smaller, measuring only 0·27 mm. in diameter. It is situated at a distance of 1·12 mm. from the anterior end. The neck is thus about 3/8 of the body length.

There is a very small prepharynx followed by a pharynx of comparatively enormous size, its dimensions being 0·31 × 0·34 mm. There is no oesophagus, the intestinal diverticula separating immediately behind the pharynx and passing out at a wide angle towards the sides of the body; they extend almost to the posterior end.

The excretory system was not observed.

The two moderately large globular testes lie in the posterior half of the body, one in front of the other and contiguous. The posterior testis is separated from the end of the body by a space equal to its diameter, which is 0·45 mm. The cirrus pouch is small and stout, lying immediately and entirely in front of the ventral sucker. It contains a comparatively large and globular vesicula seminalis but other details of its internal structure were obscured by the presence of eggs in the genital sinus. The genital aperture is in the middle line a little in front of the intestinal bifurcation.

The ovary is situated just in front of, and contiguous with, the anterior testis, but displaced towards the right side. It is transversely oval and measures about 0·37 × 0·26 mm. The yolk glands are very voluminous filling up the greater part of the posterior half of the body and extending along each side to the level of the middle of the pharynx. The follicles are somewhat small and are arranged in a peripheral layer, which in front of the ventral sucker extends right across the body dorsally but only for a short distance ventrally. At the level of the ovary the ventral layer begins to extend in towards the middle line overlapping the edges of the ovary and anterior testis and almost completely covering the posterior testis. Behind the latter the follicles from each side merge and completely fill the post-testicular space. There is no receptaculum seminis but the initial two or three convolutions of the uterus are packed with sperm. No Laurer's canal was observed. The uterus fills up the region between the ovary and the ventral sucker, and is confined within the space bounded by the intestinal diverticula. The eggs do not exceed 100 in number. They are brownish yellow and of moderate size, measuring 0·078–0·084 × 0·046–0·050 mm.

The systematic position of this species is a matter of some difficulty. It is obviously related to the groups of which *Lepocreadium* and *Stephanochasmus* are the chief representatives, but it presents such a combination of the characters of the two groups that it is difficult to decide to which it is more nearly related. Thus, for instance, it has the reduced ventral sucker of the Lepocreadiinae but, on the other hand, it lacks the vesicula seminalis externa and the receptaculum seminis of this group. Again it has the uterine receptaculum seminis and the large pharynx characteristic of the Stephanochasminae but it lacks the crown of cephalic spines. For the present it must be regarded as an intermediate type. It does not appear to be closely allied to any other known form. Its generic characters may be summarized as follows:

Lepidauchen n. g.

Body broad and flat; covered with spines anteriorly. Oral sucker much larger than ventral, which lies in front of the middle of body. Prepharynx short, pharynx very large, oesophagus absent. Genital aperture median, near intestinal bifurcation. Cirrus pouch short and stout. Receptaculum seminis absent. Otherwise as in Allocreadiinae

Type species: *L. stenostoma* from intestine of *Labrus berggylta.*

Hemipera ovocaudata n. g., n. sp.

(Figs. 4, 5.)

This peculiar and interesting form was found in the stomach of four out of 24 specimens of *Lepadogaster gouanii.* In all but one case only a single immature example was obtained; in the fourth three examples occurred, two of which contained eggs.

The species is small and delicate, elongated and sub-cylindrical in shape, with somewhat pointed ends. The largest specimen measured 1·54 mm. in length with a breadth of 0·56 mm. across the ventral sucker. The surface of the body is smooth and has no cuticular spines.

Both suckers are globular. The oral sucker is subterminal and measures 0·22 mm. in diameter. The ventral sucker is much larger, measuring 0·40 mm., and is situated at a distance of 0·87 mm. from the anterior end of the body, *i e.* distinctly behind the middle.

Contiguous with the oral sucker is a medium sized pharynx having a diameter of 0·066 mm. It is followed by a very short oesophagus. The diverticula pass out at right angles to the oesophagus but after a short distance bend abruptly backwards and run down the sides of the body to the posterior end, their ends being very close together. The excretory vesicle resembles that of *Derogenes* and the Hemiuridae. The median stem, which is very narrow, divides into two near the posterior border of the ventral sucker. The paired limbs pass out towards the sides of the body but at the level of the pharynx they turn in and unite dorsal to the pharynx.

The testes are symmetrically situated near the posterior end of the body, from which they are separated by a space equal to half their length. Each slightly overlaps the corresponding intestinal diverticulum and they are separated from each other by a narrow space through which runs the excretory vesicle. They are elongated oval bodies, measuring 0·22 × 0·14 mm. Their long axes are slightly oblique.

The genital aperture lies in the middle line immediately behind the intestinal bifurcation. The somewhat elongated cirrus pouch contains only the pars prostatica and the ductus ejaculatorius. The vesicula seminalis lies entirely outside the pouch, with which it is connected by a fairly long duct, which is bent up alongside the pouch. The pouch is divided into two parts by a distinct constriction about its anterior third. The posterior part is entirely filled with the pars prostatica. The vesicula seminalis, which lies alongside the cirrus pouch and somewhat behind it, is a little smaller than the posterior portion of the pouch.

The ovary lies to the right of the middle line immediately behind the ventral sucker. It is a small globular body of 0·09 mm. diameter. The yolk glands lie on either side of it and a little in advance. Both overlap the intestinal diverticula. Each consists of a compact ovoid mass of follicles, which is somewhat larger than the ovary but smaller than the testes (0·15 × 0·08 mm.). The yolk ducts pass behind the ovary and a fairly conspicuous yolk reservoir is formed. There is a large shell gland and a small receptaculum seminis but Laurer's canal is apparently absent. It is somewhat doubtful if the receptaculum seminis is a constant structure. In the living specimen it appeared at times to be quite distinct but sometimes it seemed to be merely a dilatation of the oviduct. In no case did it contain more than a few spermatozoa. In the preserved specimens its presence could not be detected. The uterus is of no great extent and the largest specimen contained less than thirty eggs. Possibly none were completely mature. The eggs were scattered around the ovary and along the left side of the ventral sucker. They are remarkable in possessing a single long filament extending from the anopercular pole. The filaments were directed backwards and to some extent intertwined. The eggs are slightly curved and measure about 0·10 × 0·027 mm. and the filaments about 0·2 mm.

The systematic position of this form will be discussed after the next species has been described.

Derogenoides ovacutus n. g., n. sp.

(Fig. 6.)

This is a form which bears a much greater resemblance to *Derogenes varicus* than the preceding species does, though it presents one or two marked features of difference. Numerous specimens were met with on one occasion in the stomach of *Trachinus draco*. The only other specimen of this fish which I have had an opportunity of examining was obtained from the North Sea and was not infected with this parasite. Along with this new species there occurred a small number of specimens of *Derogenes varicus.*

It is a rather small form, mature specimens measuring only 0·6–0·9 mm. in length. 0·6 mm. appears to represent its minimum adult length as all the specimens below that were immature. Its shape is elongated, sub-cylindrical with rounded ends. The greatest breadth in an average specimen (0·73 mm. long) is 0·18 mm. The breadth, however, is fairly uniform. The cuticle is smooth and unarmed.

The oral sucker is subterminal with a small fleshy lobe projecting in front of it. It is globular with a diameter of 0·066 mm. The ventral sucker is also globular with a diameter of 0·123 mm. It lies 0·34 mm. from the anterior end, *i.e.* a very little in front of the middle of the body.

Contiguous with the oral sucker is a moderately large pharynx, measuring 0·039 mm. in diameter. The oesophagus is short and in no case longer than the pharynx. The intestinal diverticula are simple and fairly straight, reaching almost to the posterior end of the body.

The excretory vesicle resembles that in *Derogenes,* the unpaired median stem dividing some distance behind the ventral sucker (between the testes) and the paired limbs uniting dorsal to the pharynx.

The disposition of the genital glands also resembles that in *Derogenes,* the testes being approximately symmetrical with a slight tendency to obliquity. They are separated from the ventral sucker by a space of about 0·04 mm. and measure 0·12 × 0·096 mm. Their axes are oblique, the anterior pole in each case being directed outwards. Immediately behind the testes lies the large transversely oval ovary, the dimensions of which are 0·07 × 0·12 mm. Behind this again lie the globular yolk glands, which are practically symmetrical. In front of the ovary and between the posterior ends of the testes lies a small receptaculum seminis. The shell gland complex is situated on the dorsal side of the ovary.

The uterus is not very voluminous and contains only about 40 eggs. These are situated for the most part behind the yolk glands. The terminal part of the uterus passes up between the testes, over the right side of the ventral sucker and unites with the proximal end of the cirrus pouch. The eggs differ from those of *Derogenes* in having the anopercular pole drawn out into a sharp point. In their passage through the uterus the pointed end is always directed backwards. They measure 0·033–0·042 × 0·015–0·019 mm., the average being 0·038 × 0·018 mm.

The terminal part of the male organs bears a general resemblance to that of *Derogenes* but differs from it in detail. It is in the first place not so elongated, the vesicula seminalis being small and globular. The pars prostatica is considerably shorter and the prostatic cells much fewer in number. The pseudo-cirrus pouch, however, is distinctly longer, being somewhat cylindrical instead of globular. The posterior end is slightly inflated. The genital aperture lies immediately over the intestinal bifurcation.

The two foregoing species present not a few features of considerable taxonomic interest. They obviously belong to the small group of forms, inhabiting the stomach, of which *Derogenes varicus* is the best known example. *Hemipera* differs from all the forms which may be included in this group by reason of the structure of its cirrus pouch, which contains not only the ductus ejaculatorius but also the pars prostatica. In all other members the latter is free. It presents the further peculiarity of having the testes situated behind the ovary. In this respect it resembles *Liocerca*, to which it is probably more closely allied than to any other genus.

Derogenoides, on the other hand, is a typical member of the group with a free pars prostatica and the testes in front of the ovary. It bears, indeed, a very close resemblance to *Derogenes*. The somewhat different structure of the terminal male organs, however, together with the more anterior position of the ventral sucker and the genital glands and the characteristically shaped eggs, appear sufficient grounds for excluding it from this genus.

The systematic position of *Derogenes* has for long been a difficulty. It was included in the family Hemiuridae by Lühe (1901) and its somewhat isolated position in this family was recognised by Odhner (1904) who suggested that a separate sub-family would probably be required for its reception. On the other hand, Looss (1907) definitely excluded it from this family. There can be little doubt, however, of its Hemiurid affinities. Its whole structure, apart from the absence of an appendix, gives evidence of this and there appears no very strong reason why it should for the present be excluded from this family. At the same time it displays considerable affinity with the Syncoeliinae and the inclusion of *Derogenes* within the Hemiuridae would necessitate the inclusion of this sub-family as well. Odhner (1911), indeed, has advocated the advisability of this step, including *Derogenes* actually within the sub-family Syncoeliinae. He extends the family moreover to include the Accacoeliinae and the group of which *Hirudinella clavata* is the chief representative. In this reconstructed family Looss's Hemiuridae takes the position of a sub-family.

In view of the great variety of structure which occurs in these forms Odhner's arguments in favour of a wider conception of the family group appear to be well founded. It is particularly in regard to the terminal part of the male organs that the finer distinctions have arisen, but it seems inadvisable to allow the consideration of these to outweigh the value of other structural features. The various modifications of the cirrus pouch which are met with in the true Hemiurids appear again in the *Derogenes* group, and if the same narrow limits of classification were adopted in this group the necessity would arise of creating a number of small sub-families and of erecting the Syncoeliinae into a separate family. I am on that account in agreement with Odhner in considering that, for the present at any rate, such a scheme of subdivision would not be advantageous.

Obviously the two genera, *Hemipera* and *Derogenoides,* must be included in the sub-family Syncoeliinae, *Derogenoides* in close relation to *Derogenes* and *Hemipera* to *Liocerca*. In the event of further sub-division eventually becoming necessary it is apparent that these two pairs would form the nuclei of smaller groups, both differing from the true Syncoeliid type in having the ends of the intestines free, and the *Liocerca-Hemipera* group being further distinguished by the inverted position of the ovary and testes.

The inclusion of these two forms within the family Hemiuridae, *sensu lat.*, involves slight modifications of Odhner's amended definition, namely: Pars prostatica usually free but not in *Hemipera*. Ova 0·015–0·100 mm., usually oval, but sharply pointed at one end in *Derogenoides,* and filamented in *Hemipera*.

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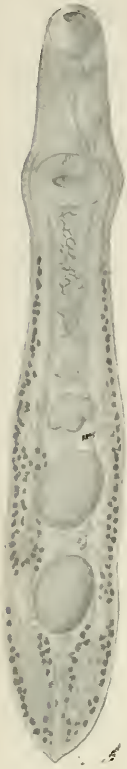
EXPLANATION OF PLATE XI.

Fig. 1. *Podocotyle syngnathi.* Ventral view. × 20.
Fig. 2. *Podocotyle syngnathi.* Cirrus pouch and vagina. × 80.
Fig. 3. *Lepidauchen stenostoma.* Ventral view. × 30.
Fig. 4. *Hemipera ovocaudata.* Ventral view. × 66.
Fig. 5. *Hemipera ovocaudata.* Shell gland complex. × 130. Semi-diagrammatic.
Fig. 6. *Derogenoides ovacutus.* Ventral view. × 150.



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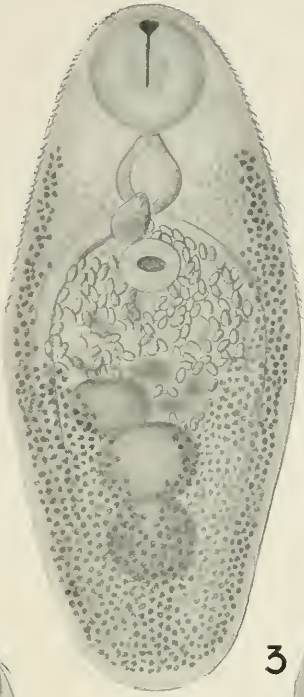

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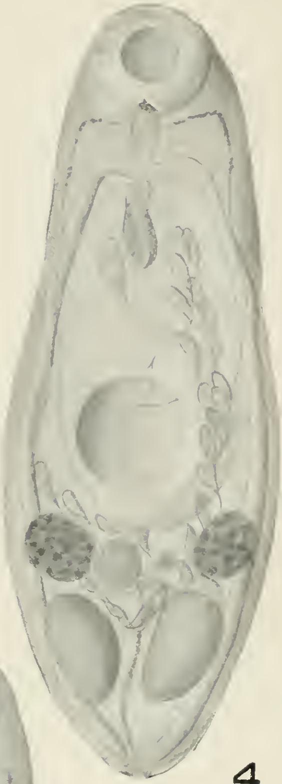

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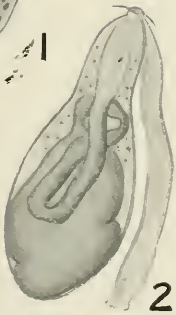

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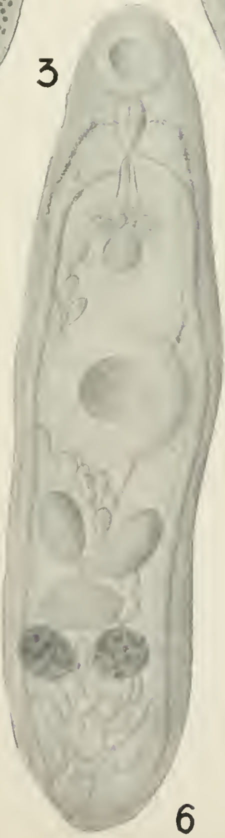

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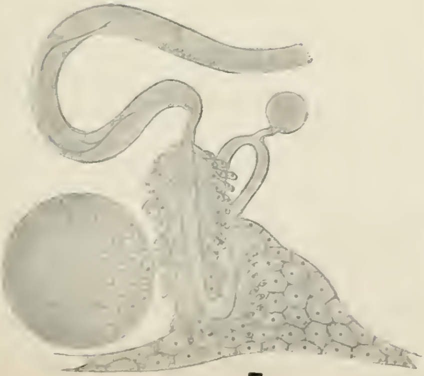

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No 3

III. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. COMPARISON OF PREPARATIONS OF GLYCOGEN FROM DIFFERENT SOURCES.

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(*Received November 23rd 1912.*)

For some time considerable uncertainty has prevailed as to whether samples of glycogen prepared from different sources are identical, the evidence on this point being in many respects contradictory. It is agreed that all have the same empirical formula $C_6H_{10}O_5$, the different results obtained by some observers being undoubtedly due to insufficient drying of their glycogen preparations. For Harden and Young [1902] found that the last traces of water can only be removed by heating to 100° C. *in vacuo* over phosphorus pentoxide, a procedure which had not been adopted by previous workers. Very divergent values have however been obtained for the specific rotation of different preparations of glycogen, the results covering a range of about 30°. Thus Cremer [1894] found as the $[\alpha]_D$ of yeast glycogen + 198·9°, while Clautriau's [1895] result was 184·5°. Harden and Young [1902] also obtained for oyster and rabbit glycogens the value 191·2° while with yeast glycogen the mean of several determinations was 198·3°, a result in close agreement with that found by Cremer. It is uncertain however whether these differences are of any real significance, the strong opalescence of glycogen solutions making necessary the use of very dilute solutions and thereby introducing a large experimental error.

Other points of difference have however been noticed by various observers, though again the results are contradictory. Cremer for example states that yeast glycogen gives a darker colouration with iodine than does oyster glycogen, while other workers have obtained exactly opposite results. In a similar way different preparations of glycogen yield solutions with varying degrees of opalescence but these variations are by no means constant either in nature or extent. At present it is impossible to explain these results and at the same time it is uncertain how far divergences of this nature imply any real differences between the respective glycogens.

A more valuable comparison was made by Harden and Young when they measured the rate of hydrolysis of different glycogen solutions by dilute acids. The course of hydrolysis was followed by estimations of the polarimetric and reducing powers of the solutions. By this method they were unable to detect any differences between rabbit, yeast and oyster glycogens and came to the conclusion that they were in all probability identical.

It seemed possible however that a study of the behaviour of various preparations of glycogen towards diastatic ferments might prove a more delicate means of detecting any difference in their constitution, for it has on many occasions been pointed out what marked influences even small alterations in the molecule may exert on the course of enzyme action. It is the results of such an investigation which are presented in this communication. Four preparations of glycogen have been employed, these being derived from (*a*) liver of dog, (*b*) liver of rabbit, (*c*) oyster and (*d*) yeast. The enzyme used was an extract of pig's pancreas, this being one of the most convenient sources for obtaining active preparations. Before making any direct comparison, the general conditions of action were studied, for although a great amount of work has been carried out on the behaviour of starch towards diastatic enzymes, the hydrolysis of glycogen has received very much less attention. These experiments will be first described.

PREPARATION OF GLYCOGEN.

A. Dog. A large dog was fed with considerable quantities of carbohydrate for forty-eight hours and then killed. The liver was rapidly removed, minced and heated on the water bath with 60 per cent. caustic potash. The glycogen was precipitated from this solution in the usual way [Pflüger, 1905] and purified by repeated reprecipitation from its aqueous solution, the first two or three precipitations being carried out with solutions rendered faintly acid with acetic acid. Glycogen was in this way obtained in considerable quantity, free from nitrogen and containing only a trace of ash. In the final stages it is frequently difficult to produce a satisfactory precipitation with alcohol alone, the addition of a small quantity of acetone in these cases, however, readily brings down all the glycogen, leaving a liquid which filters well.

B. Rabbit. The method was the same as that described above, a dozen rabbits being used for each preparation. They were previously fed on large quantities of carrot.

C. Oyster. A gross of oysters (1500 grms.) were worked up by Pflüger's method and yielded 120 grms. purified glycogen.

D. Yeast. The glycogen was separated and purified from yeast gum by the method recently described by Harden and Young [1912] with the exception that yeast juice was employed as the starting material. Large quantities of yeast juice were boiled, filtered and the filtrate precipitated with alcohol. The precipitate was then heated on the boiling water bath with 60 per cent. caustic potash to remove protein, the solution diluted and again precipitated with alcohol. After thorough washing with 50 per cent. alcohol and reprecipitation, the glycogen was purified from yeast gum by saturation of the solution with ammonium sulphate, which precipitates the glycogen but not the gum. This process was repeated three times and the glycogen then dialysed to remove the ammonium sulphate. The solution was then again precipitated with alcohol and the further purification carried out as in the final stages described above. The preparation is extremely tedious and the yield very variable according to the history of the yeast employed.

The ash content of the four specimens was as follows. Dog glycogen, 0·38 per cent. Oyster, 0·2 per cent. Rabbit, 0·51 per cent. Yeast, 0·87 per cent. The reaction of the two latter was faintly alkaline, while the dog and oyster preparations both yielded slightly acid solutions.

PREPARATION OF ENZYME.

100 grms. of pig's pancreas were minced and well ground up with half the weight of sand. Sufficient kieselguhr was added to form a fairly stiff mass which was then pressed out in a hydraulic press. The extract obtained in this way after filtration yielded a clear yellow-brown liquid which was extremely active. It was usually diluted to five or ten times its volume before use.

EXPERIMENTAL METHODS.

The course of the hydrolysis can be followed in several ways: (i) estimations of the residual glycogen, (ii) determinations of the optical activity, and (iii) estimations of the reducing power of the solution after known intervals of time. The first of these methods however is unsatisfactory owing to the fact that some of the higher dextrins are also precipitated by alcohol as well as the glycogen, for a precipitate is still obtained in this way some time after the disappearance of the iodine reaction. The optical activity of the solution also is due to a complex mixture of substances, the nature of which has not been thoroughly worked out and hence in the majority of the following experiments, the hydrolysis has been followed by estimations of the reducing power alone, though this method is not altogether free from the same objection. The latter were carried out by Bertrand's method and the reducing power calculated as maltose, preliminary experiments having shewn that under the conditions employed very little if any hydrolysis of the maltose into glucose took place. The reducing power of a maltose solution of known strength was first determined and from the results obtained, a curve constructed from which the maltose corresponding to any found weight of copper could be read off.

The experiments were all carried out in flasks of Jena glass which were used for no other purposes. In most cases toluene was added to the solutions to prevent bacterial contamination.

Hydrolysis of Glycogen by an Extract of Pig's Pancreas.

To study the general course of the hydrolysis, 500 c.c. of a 1 per cent. solution of oyster glycogen were incubated with 2·5 c.c. of a pancreas extract with the addition of 5 c.c. toluene as antiseptic. The glycogen and enzyme solutions were each brought to the temperature of the bath (37°) before mixing and then after known intervals of time, samples of the liquid were removed and the following estimations made.

A. Reducing power.

B. Matter precipitable by alcohol (glycogen and higher dextrins). The precipitation was carried out by adding two volumes of alcohol. At the same time the reaction of the solution towards iodine was observed.

The results are collected in the following table.

TABLE I.

Hydrolysis of Glycogen by Pancreatic Diastase.

Time	Duration of action	Wt. of ppt. thrown down by 2 vols. alcohol	Maltose	Iodine reaction	Percentage hydrolysis
12.30	0	4·702 grms.	—	Dark red brown	0
12.35	5 mins.	—	0·223 grm.	Red brown	4·45
12.45	15 ,,	3·49 ,,	0·664 ,,	,,	13·32
1.00	30 ,,	2·74 ,,	1·156 grms.	,,	23·2
1.15	45 ,,	2·30 ,,	1·471 ,,	Pale red brown	29·5
1.30	60 ,,	1·83 ,,	1·714 ,,	Very faint	34·4
2.0	90 ,,	1·48 ,,	2·003 ,,	None	40·2
2.30	2 hrs.	1·20 ,,	2·145 ,,	—	43·0
3.30	3 ,,	—	2·292 ,,	—	46·0
4.30	4 ,,	0·65 grm.	2·362 ,,	—	47·4
5.30	5 ,,	—	2·434 ,,	—	48·8
9.30	9 ,,	Estimation spoilt	2·550 ,,	—	51·2

In Fig. 1 the results are shewn graphically, curve A representing the degree of hydrolysis and curve B the residual glycogen and dextrins precipitable by two volumes of alcohol. It will be seen that the action commences at a high initial velocity and proceeds for a very brief period in a linear direction. By the time the iodine reaction has disappeared, the rate has slowed down considerably and finally a long period of extremely slow hydrolysis follows. It would appear that the rapid initial stage is chiefly concerned with the conversion of the glycogen into dextrins with simultaneous production of sugar. These dextrins, which are very resistant to further hydrolysis, are then slowly broken down to sugar. It is obvious however that in the above experiment this second stage would never have reached completion and this seems to be the general rule unless very high enzyme concentrations are employed. The iodine reaction disappeared when about 40 per cent. of the glycogen had been completely hydrolysed to maltose. The action in short closely resembles the hydrolysis of starch but is slower and less complete.

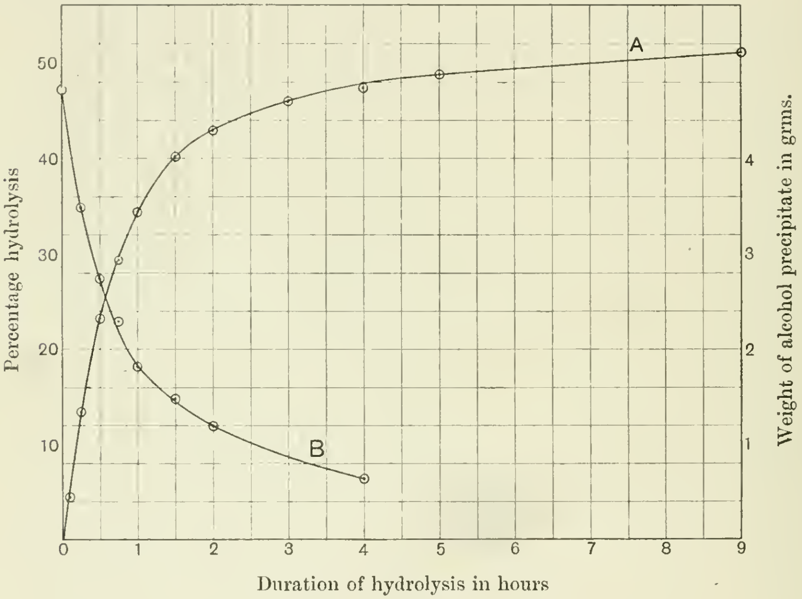


Fig. 1.

INFLUENCE OF TEMPERATURE.

A series of flasks each containing 50 c.c. of 1 per cent. glycogen were incubated with 0·5 c.c. enzyme solution, at temperatures varying from 25° to 50°. Thirty minutes after the addition of the enzyme, the action was stopped by adding 5 c.c. of N. KOH, the liquid was at once cooled and the volume made up to 100 c.c. The reducing power of 20 c.c. of this dilution was then estimated. The results are shewn below in Table II and graphically in Fig. 2. For the sake of comparison the results of a starch hydrolysis with the same enzyme preparation are given also.

TABLE II.

Influence of Temperature.

Temperature of incubation	A. Glycogen		B. Starch	
	Total maltose	Percentage hydrolysis	Total maltose	Percentage hydrolysis
25·5°	0·1070	20·2	—	—
27·5°	—	—	0·1310	24·7
34°	0·1212	22·9	—	—
37°	0·1375	25·9	0·1545	29·2
39·5°	0·1330	25·1	—	—
42°	0·1310	24·7	0·1700	32·1
46°	0·1260	23·7	0·1895	35·7
50·5°	0·1190	22·5	0·1760	33·2

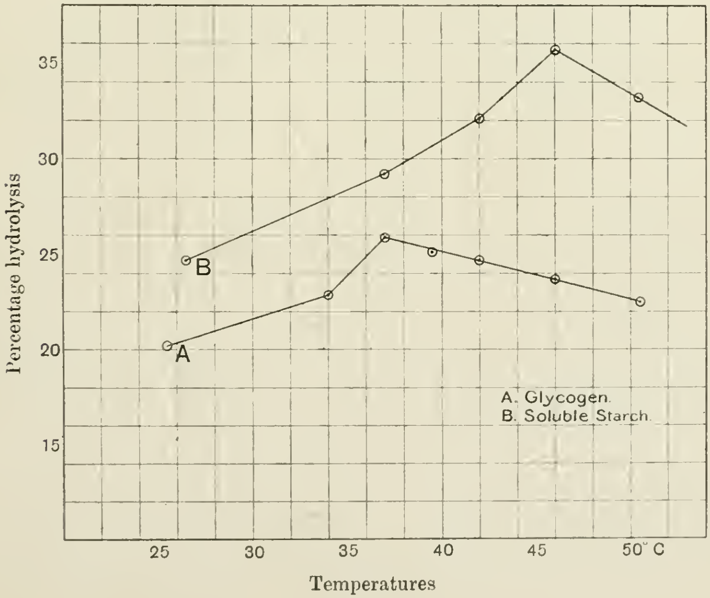


Fig. 2.

Hence with the enzyme employed in these experiments, the optimum temperature for glycogen hydrolysis was about 37° C. and this value was unchanged by substituting different preparations of glycogen. For starch hydrolysis on the other hand the optimum temperature found with the same enzyme solution was 46° C. This result therefore lends some support to the hypothesis that the two actions are brought about by two distinct enzymes.

INFLUENCE OF ENZYME CONCENTRATION.

Three dilutions of the enzyme solution were prepared and 0·5 c.c. of each added to a series of flasks each containing 50 c.c. of a 1 per cent. glycogen solution (oyster) + 1 c.c. toluene and previously brought up to 37°. Altogether three flasks were used with each dilution.

After 5, 15, and 25 minutes' incubation one flask of each series was removed from the bath and the action stopped by the addition of 5 c.c. N. alkali. The reducing power of each solution was then determined and the results are shewn in Table III below.

TABLE III.

Influence of Enzyme Concentration.

Each flask contained 50 c.c. 1 per cent. glycogen + 1 c.c. toluene + 0·5 c.c. enzyme.

		Reduction after time given, mgrms. Cu from 20 c.c. liquid		
Exp.	Dilution of enzyme solution	5 mins.	15 mins.	25 mins.
A	1/10	7	21·5	32·0
B	1/20	3·35	10·0	—
C	1/40	1·7	5·2	9·0

These results shew very clearly two things. (i) Under the above conditions the rate of hydrolysis is directly proportional to the concentration of enzyme, and (ii) the action is a linear one, the reduction after 15 minutes' being almost exactly three times that after five minutes' incubation and so on. These results only obtain however when very dilute enzyme preparations are used and during the initial part of the experiment—in other words while there is a considerable excess of unchanged glycogen. Similar results have been obtained by Evans [1912] in the hydrolysis of starch by saliva.

When stronger preparations of enzyme are employed the above relations can only be observed for a very brief period of the hydrolysis, but another point now becomes more noticeable. With dilute enzyme preparations, the action comes to an end long before all the glycogen has been completely converted into maltose. This is apparent in the results quoted in Table I where it will be noticed that after nine hours there was only a 51 per cent. hydrolysis and the action had practically come to an end. When however higher concentrations of enzyme are employed, the end point of the reaction is moved very much nearer to complete hydrolysis as will be seen from the experiments described below.

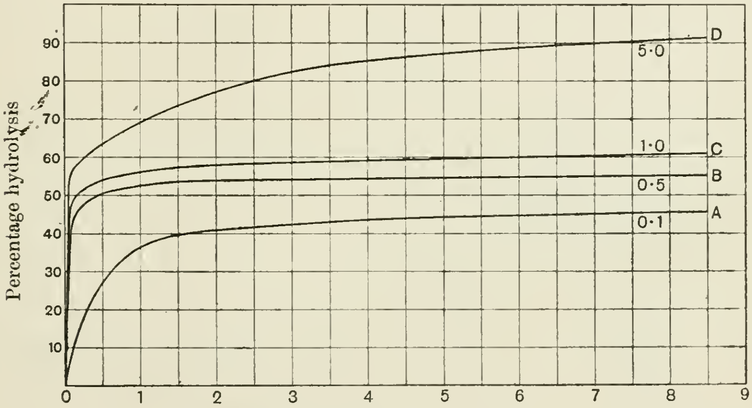
A series of flasks was incubated at 37°C. containing the following solutions:

A. 100 c.c. 1 per cent. glycogen solution + 4·9 c.c. H_2O + 0·1 c.c. enzyme + 1 c.c. toluene.

B. 100 c.c. 1 per cent. glycogen solution + 4·5 c.c. H_2O + 0·5 c.c. enzyme + 1 c.c. toluene.

C. 100 c.c. 1 per cent. glycogen solution + 4·0 c.c. H_2O + 1·0 c.c. enzyme + 1 c.c. toluene.

D. 100 c.c. 1 per cent. glycogen solution + 0 c.c. H_2O + 5·0 c.c. enzyme + 1 c.c. toluene.



Duration of hydrolysis in hours

Fig. 3.

The total volume in each case was the same, the only difference being the concentration of enzyme. After 8½ hours the action was almost over in all four solutions and the amount of hydrolysis which had taken place was as follows:

A, 45·6 per cent. B, 55·1 per cent. C, 60·7 per cent. D, 91·3 per cent. The complete course of the hydrolysis in each case is shewn in Fig. 3.

Very similar results were obtained by Philoche [1908] with taka diastase and it is not quite clear what interpretation is to be placed upon them, for, as will be seen, there appears to be no direct relationship between the concentration of enzyme and the final degree of hydrolysis. The influence of the products formed during the hydrolysis may be partially responsible but judging from experiments described later, it is unlikely that this is the sole cause. The incompleteness of the hydrolysis cannot be ascribed to the destruction of the enzyme, for the latter after 20 hours' incubation rapidly attacked a second quantity of glycogen.

INFLUENCE OF GLYCOGEN CONCENTRATION.

Unless very low concentrations are used—less than 0·5 per cent. with the strength of enzyme I have generally employed—the *initial* rate of hydrolysis is practically unchanged by increasing the glycogen concentration. In the weaker solution, however, the rate naturally begins to fall at an earlier period owing to the glycogen being more rapidly exhausted. These points are illustrated by the results shewn in Table IV.

TABLE IV.

Influence of Glycogen concentration.

Time after addition of enzyme	Reduction, mgrms. Cu from 20 c.c. liquid		
	A. 1 % glycogen	B. 2 % glycogen	
5 mins.	9·0	9·0	Rates equal.
15 ,,	26·8	27·0	
30 ,,	45	52	Rate in A falling, B practically unchanged.
1 hr.	66	89	Both rates falling, but more rapidly in A.
4 hrs.	92	147	
20 ,,	108	188	Action at an end in both.
% hydrolysis	49·6	43·5	

INFLUENCE OF PRODUCTS.

As previously stated, unless high concentrations of enzyme are employed the hydrolysis is not complete. In order to see whether this was due to the influence of the products the following experiments were carried out.

Influence of Maltose. In a preliminary experiment the action had stopped when about 50 per cent. of the glycogen had been hydrolysed to maltose, i.e. when the solution contained rather over 0·5 per cent. of maltose. This amount of maltose was therefore added to one flask at the beginning of the experiment and the rate of hydrolysis compared with one containing no sugar at the beginning. The composition of the two solutions was as follows:

Flask A. 100 c.c. 1 per cent. glycogen + 1 c.c. toluene + 0·5 c.c. enzyme.

Flask B. 100 c.c. 1 per cent. glycogen solution containing 0·5 grm. maltose + 1 c.c. toluene + 0·5 c.c. enzyme.

TABLE V.

Influence of Maltose.

Time after addition of enzyme	A. Glycogen alone	B. Glycogen + maltose		
	Mgrms. Cu from 20 c.c.	Mgrms. Cu from 20 c.c.	Cu due to added maltose	Cu due to maltose formed
15 mins.	27	136	108	28
30 ,,	46	154	,,	46
1 hr.	66	173	,,	65
2 hrs.	80	188	,,	80
4 ,,	92	199	,,	91
8 ,,	100	207	,,	99
20 ,,	108	212	,,	104
$^0/_0$ hydrolysis	49·6			47·6

It will be seen from these results that the rate of hydrolysis was practically unchanged by the maltose added and the final degree of hydrolysis lowered by only two per cent. The dextrins formed during the course of the reaction, however, seemed to have some retarding influence, this being illustrated by the following experiment.

Influence of Dextrin. 100 c.c. of 2 per cent. glycogen were incubated at 37° with 0·5 c.c. enzyme until the solution no longer gave the iodine reaction. The action was then stopped by heating the flask in a boiling water-bath. The solution thus obtained was free from glycogen and in addition to the dextrins contained about 0·9 per cent. of maltose. It is referred to below as "products of hydrolysis." The following mixtures were then made up and incubated at 37°.

A. 50 c.c. 2 per cent. glycogen + 50 c.c. "products of hydrolysis" + 1 c.c. toluene + 0·5 c.c. enzyme.

B. 50 c.c. 2 per cent. glycogen + 50 c.c. H_2O + 1 c.c. toluene + 0·5 c.c. enzyme.

Incubation was continued for six hours, the reducing power of the two solutions being estimated from time to time. The results are collected in Table VI.

TABLE VI.

Influence of Products of Hydrolysis.

	B. Glycogen alone	A. Glycogen + products		
Time after addition of enzyme	Reduction, mgrms. Cu from 20 c.c. liquid	Reduction, mgrms. Cu from 20 c.c. liquid	Cu due to sugar present at start	Cu due to maltose formed
15 mins.	26	110	84	26
30 ,,	45	128	,,	44
1 hr.	66	149	,,	65
2 hrs.	81	159	,,	75
4 ,,	90	166	,,	82
6 ,,	97	170	,,	86
% hydrolysis	44·6			39·3

In this case although there was again no difference in the initial rates, after one hour the hydrolysis in A began to lag behind that in B and after six hours only 39·3 per cent. of the glycogen in A had been hydrolysed as against 44·6 per cent. in B. Moreover in A the action had almost stopped while in B it was still proceeding at a fair rate. We have here then a marked hindering action which must apparently be due to the dextrin-like substances formed during the hydrolysis.

INFLUENCE OF ACIDITY OR ALKALINITY.

The ash almost always present in samples of glycogen consists to a large extent of phosphates and according to the nature of these phosphates the solution may have either an acid or alkaline reaction. Before comparing different samples of glycogen it was therefore necessary to determine to what extent this difference in reaction would influence the rate of hydrolysis. It is obvious moreover that the optimum dose of acid will vary according to the source and proportion of the enzyme used, for the latter as well as the solution acted on may contain substances capable of neutralising some of the acid. On the other hand the optimum hydrogen ion concentration is a constant independent of these factors. In the latter case we obtain a measurement of the true optimum reaction of the solution, while in the former we have only an apparent value, the degree of accuracy of which will depend on the amount and nature of the salts present in the solution. Thus Sörensen [1909] working with three different preparations of invertin found the optimal dose of sulphuric acid (apparent acidity) to be very different for the three preparations but the optimum hydrogen ion concentration (true acidity) was the same in each case. Hence in all the following experiments the reaction of the solution has been determined by measurements of the hydrogen ion concentration.

At first attempts were made to do this by means of the colorimetric method described by Sörensen. The strong opalescence of the glycogen solutions however rendered this method impracticable and hence the electrical method has been used throughout. The apparatus employed was that of Michaelis and Rona [1909] which is especially suitable since only small quantities of solution are required. In this method, the electrodes are kept in a still atmosphere of hydrogen and connection is made between the two cells by means of a tape soaked in a solution of potassium chloride. By making two determinations using different concentrations of KCl, e.g. 1·75 N. and 3·5 N., the contact potential due to the salt can be calculated and corrected for and, as equilibrium is established almost immediately, the method is both rapid and convenient. The determinations were all made at 37°, the temperature at which the hydrolyses were carried out, the two operations being conducted in the same thermostat. In each case the standard solution against which the glycogen solutions were compared was N/10 HCl and from the value of the E.M.F. thus set up and determined, the hydrogen ion concentration was calculated in the usual way[1].

The latter is denoted by the sign p_{H}, this being the logarithm of the reciprocal value of the factor of normality. p_{H} being known, the hydroxyl ion concentration can be calculated from the dissociation constant of water. By graphical interpolation from a series of determinations by Lundén [1907], the value at 37° has been taken as $2{\cdot}45 \times 10^{-14} = 10^{-13{\cdot}61}$. Hence in a N/100 solution of hydrogen ions where $p_{\mathrm{H}} = 2$ the hydroxyl ions will have a concentration at 37° of $10^{-11{\cdot}61}$ and so on. In the same way at absolute neutrality the concentration of both ions will be $10^{-6{\cdot}8}$.

DETERMINATION OF OPTIMUM REACTION.

To a series of flasks each containing 100 c.c. of a 1 per cent. glycogen solution, were added varying amounts of N/100 NaOH or H_2SO_4. The total volume of the solutions, however, was kept the same in every case. Each flask was then in turn placed in the bath at 37° and when the solution had acquired that temperature 0·5 c.c. of a dilute enzyme preparation was added.

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[1] A full account will be found in Sörensen's paper [1909] where he also discusses the possible sources of error in the method.

After thorough mixing, a sample was removed and the hydrogen ion concentration at once determined. Thirty minutes after the addition of the enzyme, a second sample was taken, the action stopped by the addition of a small quantity of N. alkali and the reducing power estimated. In this way the true reaction and the degree of hydrolysis was determined in each case. This process was repeated with all four preparations of glycogen. In these experiments no toluene or other antiseptic was used, Sörensen having found that these substances interfere somewhat in the hydrogen ion determinations, equilibrium not being so readily established. As the duration of each experiment, however, was only 30 minutes it is highly improbable that this in any way influenced the results. These are collected in Table VII and also shewn graphically in Fig. 4.

TABLE VII.

Influence of hydrogen ion concentration on the rate of hydrolysis.

Experiment	Source of glycogen	C.c. of N/100 acid or alkali added	E.M.F. volts	p_H	Reduction, mgrms. maltose in 20 c.c.	% hydrolysis in 30 mins.
1	Dog liver	3·0 H_2SO_4	0·2055	4·43	7·37	3·7
2	,,	1·0 ,,	0·2306	4·84	33·5	16·8
3	,,	0·5 ,,	0·2754	5·57	52·9	26·6
4	,,	0 ,,	0·2940	5·87	59·0	29·6
5	,,	0·25 NaOH	0·3018	6·00	61·4	30·8
6	,,	0·5 ,,	0·3217	6·32	60·3	30·3
7	,,	1·0 ,,	0·3410	6·63	54·0	27·1
8	,,	3·0 ,,	0·3755	7·19	37·1	18·6
9	,,	6·0 ,,	0·4078	7·72	12·1	6·1
14	Oyster	0·5 H_2SO_4	0·2260	4·76	28·0	14·1
15	,,	0 ,,	0·2730	5·53	40·4	20·3
16	,,	0·5 NaOH	0·2870	5·76	44·2	22·2
17	,,	1·0 ,,	0·3019	6·00	44·4	22·3
18	,,	3·0 ,,	0·3322	6·49	32·8	16·5
19	,,	6·0 ,,	0·3980	7·56	10·7	5·4
22	Rabbit liver	6·0 H_2SO_4	0·2492	5·14	4·8	2·4
23	,,	5·0 ,,	0·2750	5·56	35·6	17·9
24	,,	4·75 ,,	0·2850	5·72	45·2	22·7
25	,,	4·5 ,,	0·3010	5·97	47·5	23·9
26	,,	4·0 ,,	0·3180	6·26	47·0	23·6
27	,,	3·0 ,,	0·3412	6·64	42·3	21·3
28	,,	0 ,,	0·3818	7·30	24·9	12·5
29	Yeast	6·0 H_2SO_4	0·2748	5·55	25·5	12·8
30	,,	5·0 ,,	0·2940	5·87	39·5	19·8
31	,,	3·0 ,,	0·3120	6·16	40·0	20·1
32	,,	1·5 ,,	0·3245	6·36	39·5	19·8
33	,,	0 ,,	0·3580	6·91	27·1	13·6

The results shewn above indicate that the value obtained for the optimum hydrogen ion concentration was practically the same with each preparation of glycogen, that from oyster however giving a slightly lower figure than the other three. The optimum reaction therefore for the enzyme I have used was just on the acid side of the neutral point, the hydrogen ion concentration corresponding to an acidity of about $N/10^6$. Alteration of the reaction in either direction produced a considerable reduction in the rate of hydrolysis especially in the case of the dog and oyster glycogens. Hence in comparing the rate of hydrolysis of different glycogen preparations it is essential to see that the reactions of the solutions are as nearly the same as possible, if the results are to be at all reliable.

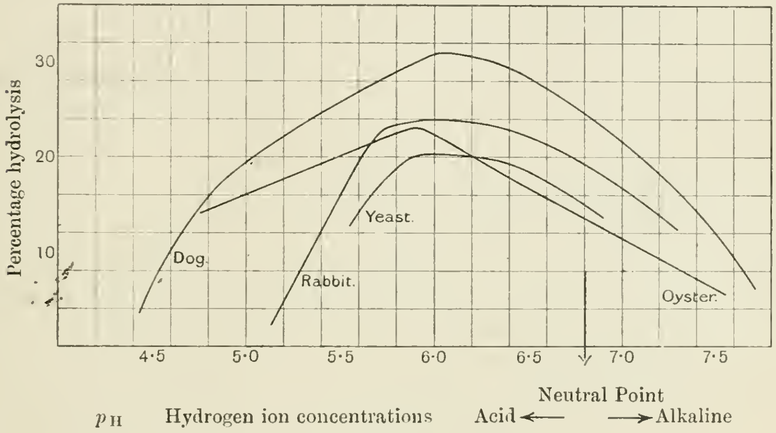

Fig. 4.

Comparison of Glycogens.

1 per cent. solutions of the four kinds of glycogen were prepared and sufficient N/100 acid or alkali added so that each solution after the addition of the enzyme was as nearly as possible at the optimum reaction. The amount to be added was estimated from the results obtained in the experiments last described, the total volume was the same in each case. Immediately after the addition of the enzyme, the hydrogen ion concentration was determined to make sure that the reaction was really correct and then after 30 minutes' hydrolysis, the action was stopped by the addition of alkali and the reducing power estimated. Both the hydrolysis and the hydrogen ion determination were carried out at 37° and no toluene was used for the reason already stated. The results are shewn in Table VIII.

TABLE VIII.

Comparison of Glycogens. I.

Source of glycogen	E. M. F. of solution against N/10 HCl at 37° C.	p_H	Mgrms. maltose per 20 c.c. after 30 mins.	Percentage hydrolysis after 30 mins.	Relative rates
Dog	0·2970 volts	5·92	46·10	23·05	100
Rabbit	0·2986 ,,	5·94	43·25	21·62	93·82
Oyster	0·2956 ,,	5·90	41·0	20·5	88·9
Yeast	0·3014 ,,	5·99	39·5	19·7	85·7

COMPOSITION OF SOLUTIONS.

A. Dog glycogen. 100 c.c. 1 per cent. glycogen + 0·25 c.c. N/100 NaOH + 4·5 c.c. H_2O + 0·5 c.c. enzyme.

B. Oyster. 100 c.c. 1 per cent. glycogen + 1·0 c.c. N/100 NaOH + 4·0 c.c. H_2O + 0·5 c.c. enzyme.

C. Rabbit. 100 c.c. 1 per cent. glycogen + 4·5 c.c. N/100 H_2SO_4 + 0·5 c.c. H_2O + 0·5 c.c. enzyme.

D. Yeast. 100 c.c. 1 per cent. glycogen + 4·25 c.c. N/100 H_2SO_4 + 0·75 c.c. H_2O + 0·5 c.c. enzyme.

There is thus quite a marked difference in the rate of hydrolysis of the four preparations of glycogen examined, the least difference being between those from oyster and yeast. A second experiment gave very similar results which are shewn in Table IX.

TABLE IX.

Comparison of Glycogens. II.

Source of glycogen	E.M.F. of solution against N/10 HCl at 37° C.	p_H	Mgrms. maltose per 20 c.c. after 30 mins.	Percentage hydrolysis	Relative rates
Dog	0·3046 volts	6·0	48·9	24·45	100
Rabbit	0·2950 ,,	5·9	46·1	23·05	94·3
Oyster	0·2901 ,,	5·8	42·4	21·2	86·7 *
Yeast	0·3112 ,,	6·1	40·45	20·2	83 †

* Solution rather too acid. † Solution rather too alkaline.

OPALESCENCE OF GLYCOGEN SOLUTIONS.

A 0·2 per cent. solution was made up of each of the four preparations of glycogen and the opalescence of these compared. This was carried out in Nessler glasses, the volume of solution being adjusted until the opalescence appeared to be the same in each case. The comparison is not easy and the experimental error is therefore large but the solution of rabbit glycogen was markedly the most opalescent of the four examined, oyster and dog glycogen being about equal and yeast less opalescent than any. The actual results obtained were as follows:

TABLE X.

Glycogen	Volumes of 0·2 solutions having equal opalescence	Relative opalescence
Rabbit	11 c.c.	4·5
Dog	30 ,,	1·7
Oyster	33 ,,	1·5
Yeast	50 ,,	1

Colouration with Iodine.

To 10 c.c. of a 0·2 per cent. solution of each of the glycogen preparations was added 1 c.c. of a 1 per cent. solution of iodine. The volume in each case was then made up to 50 c.c. and the colours compared in Nessler glasses with the following results:

TABLE XI.

Glycogen	Volumes of solutions giving equal depth of colour	Relative strength of colouration
Rabbit	10·5 c.c.	4·7
Dog	19 ,,	2·6
Yeast	32 ,,	1·5
Oyster	50 ,,	1

Thus both as regards opalescence and the colouration with iodine there is a distinct difference between the four glycogen solutions.

Summary and Conclusions.

The conditions under which glycogen is hydrolysed by a pancreatic extract (pig) have been studied with the following results:

1. The hydrolysis at first proceeds with great rapidity, the glycogen being quickly converted into dextrins with simultaneous production of maltose. The further breaking down of these dextrins takes place with extreme slowness and is as a rule incomplete.

2. The optimum temperature for glycogen hydrolysis of the enzyme employed in these experiments was 37°. Below this there is a rapid fall in the rate, between 37° and 50° this is less marked. With starch the optimum temperature for the same enzyme preparation was 46°.

3. While excess of glycogen is present, the rate of change is directly proportional to the enzyme concentration and the action is a linear one.

4. With low enzyme concentrations, the action is by no means complete; the degree of hydrolysis, however, rises with increasing concentration of enzyme though not in the same ratio.

5. The concentration of the glycogen solution has little influence on the initial rate of hydrolysis unless very low concentrations (less than 0·5 per cent. in my experiments) are employed.

6. The action is hindered to a small extent by the products of hydrolysis, the dextrins probably being the interfering substances.

7. The action is favoured by the presence of small traces of acid, the optimum reaction determined with several preparations of glycogen being a hydrogen ion concentration of 10^{-6} (at neutrality this value is $10^{-6\cdot8}$ at 37°).

8. Samples of glycogen prepared from (i) dog, (ii) rabbit, (iii) oyster, and (iv) yeast are hydrolysed at different rates when compared at the optimum hydrogen ion concentration. Taking dog glycogen as 100, the relative rates of hydrolysis found are, rabbit 94, oyster 88, and yeast 84.

9. The degree of opalescence and the colouration with iodine exhibited by glycogen solutions vary with the source from which the glycogen has been obtained.

10. In view of the above points of difference it is probable that the four preparations of glycogen examined are not identical in constitution, though it is possible these differences might be caused by variations in the colloidal state of the four glycogen solutions. If, however, the glycogens are distinct, the diastatic enzymes obtained from different animals should also be specific and experiments are in progress to test this point.

My thanks are due to Professor Arthur Harden, F.R.S. and to Dr H. MacLean for much useful advice during the course of this research.

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V. *Notes on the Life-History of* Trypanosoma gambiense, *with a Brief Reference to the Cycles of* Trypanosoma nanum *and* Trypanosoma pecorum *in* Glossina palpalis.

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INTRODUCTION.

Among the mammalian trypanosomes carried by *Glossina palpalis*, *T. gambiense* may be taken as an example affording, as it were, a basis for the study of the group. In many ways the conditions are very clear, and no very serious difficulties on the score of technique or scarcity of parasites are met with at any point in the *Glossina* cycle.

In this paper I have considered the cycle of *T. gambiense* in detail, and have treated *T. pecorum* and *T. nanum* comparatively and much more briefly. I am indebted to Dr. H. L. DUKE for the material of *T. pecorum* and *T. nanum* dealt with.

The material of *T. gambiense* was derived from a large number of experiments carried out in the Mpumu Laboratory in 1911 and 1912. The stages in *Glossina palpalis* were obtained from the study of the conditions in more than 200 infected flies, which were killed or died at different periods of the cycle. The advantage of so large a number is obvious, in that it eliminates casual variation. A considerable number of different strains of *T. gambiense* were used; thus, two separate strains derived from wild fly caught in Chagwe, a strain derived by direct injection into a monkey from an antelope infected with a human strain, this same strain after transmission by flies to clean monkeys, antelope strains of human origin passed by fly to clean monkeys, and a number of indifferent Uganda mentioned above, I have been led to the following interpretation of the cycle in the vertebrate. As here given, the account appears as a set of unsupported statements, and the reader must be referred to the earlier paper for the evidence upon which they are based :—

The short forms, 13 to 20 microns in length are the "adult" blood type—this term being used to indicate that the form in question has the longest duration in time in the endogenous cycle, and appears to be the type from which the individuals capable of carrying on the cycle in the invertebrate host are derived. The blood of a monkey is infective to *Glossina* only so long as this form is present in sufficient numbers and in an appropriate physiological state—*i.e.*, not suffering from the exhaustion which seems to overtake the flagellates at certain times in very numerous infections. From these short forms there arise by growth the intermediate individuals, which are an ill-defined and quite artificial group, chiefly to be recognised by the fact that they have increased in length and have a longer free flagellum, but are of much the same average breadth as the short forms, namely 2 to 2·5 microns. The long slender forms are derived from the intermediate type, and are simply the next step towards the division forms, which are the culminating stage of this whole process. The long slender forms are the individuals about to divide. It is incorrect to speak of three types of trypanosome; strictly speaking, it is a somewhat arbitrary attachment of names to the different stages by which the short forms proceed to division. The products of division give rise once more, directly or indirectly, as the case may be, to the adult type.

Another feature equally well known in the history of *T. gambiense* is the fluctuation in the number of trypanosomes to be seen in the blood. This has also been treated exhaustively elsewhere; it suffices to say here that a complex of circumstances, occurring at quite irregular and apparently incalculable intervals in the blood of an untreated vertebrate, brings about the disappearance of the vast majority of the parasites from the peripheral blood. The duration of the depressed period is very variable, and the reappearance of the flagellates is always accompanied by division in the peripheral blood. The percentage of division bears, moreover, an obvious relation to the rapidity of the rise in number. An important feature of the depressed period is that, from the completion of the fall in numbers, which is usually rapid, until the first beginnings of the following rise, the blood is infective to fly, and the few surviving trypanosomes are of the short type.

The mechanism of the destruction is difficult to follow, but a certain amount of trypanolysis seems to occur in the blood. The liver and lungs have shown, in the case of a very teeming infection investigated at this period, both degenerative forms and what one is inclined to call involution-phases (fig. 39B). The liver of this monkey (633) showed many rounded-off individuals, possessing a trophonucleus and a kinetonucleus, but no flagellar apparatus. These forms seemed for the most part to be between the cells, and only in a few instances to be inside the cell—but this is a point very difficult to determine accurately in smear-preparations. In addition to the rounded-off specimens, there were many trypanosomes of degenerate appearance. It is impossible to state what the fate of the rounded individuals may be. The infectivity of the blood during the depressed period and the failure to find multiplicative intracellular stages, coupled with the gradual nature of the rise and the high percentage of dividing individuals in the peripheral blood during this increase, are all arguments against the form under discussion playing any serious part in the reappearance of the typanosomes. These forms were not to be found in two late and chronic infections investigated, nor were they observed in a rather interesting case of an infection which had been a very teeming one, and which had just begun to take on the more chronic characters. This monkey died apparently from exposure during a severe storm, in which the animal had refused to stay in its box. It was observed when just dead, and the smears were taken at once. At the time of death, the infection was showing its first longer period of low numbers; it had been in a swarming condition only 10 days prior to the taking of the films. The infection had, of course, shown the usual short depressions, but these had been less frequent than is typical of the progress of affairs in monkeys.

It is impossible to dismiss altogether the consideration that the rounded forms may be latent individuals (capable of multiplication and further activity) lying in small numbers in the liver and lung, though their persistence in the latter seems less probable. Nevertheless, the three cases just cited do not bear this out, and the evidence, so far as

I have been able to see, is all in favour of the rounded forms being destined to destruction. The point must, however, be borne in mind, as the possible survival of latent forms, however scarce, in contact with or enclosed in the cells of any part of the body is exceedingly important from the therapeutic point of view.

The question of a sexual interpretation of the polymorphism of *T. gambiense* has very frequently been raised, and has, indeed, already passed into the nomenclature adopted by many workers, so that one is constrained to give it a consideration it does not really deserve on the strength of its merits as a scientific hypothesis. The interpretation put upon the appearances is that the long slender forms are males, the short forms females, and the intermediate individuals are indifferent, or simply non-sexual forms This is based primarily on a vague analogy with the life-cycle of the malarial parasite, and on the supposition that the long and short forms play the part of gametes or gametocytes in the transmitting host.

All the evidence brought to light by the study of the endogenous cycle is in absolute opposition to the sex-interpretation above sketched, and it may be pointed out that it is direct evidence of a nature easily observed by a careful study of the successive stages of the blood-cycle. The results of a long series of feeding experiments carried out on consecutive days with *G. palpalis* have further shown that it is on the presence of the short forms that the infectivity of the blood depends. It must be noted here that the distinction between the broad and slender forms is merely a physiological one, and is apparently entirely in respect to the process of division. The reason why it is from among the broad forms that the survivors in the fly are drawn is most probably that they are in a more stable condition, and therefore more capable of resisting the change of environment. Dismissing, then, the division of the long and short forms in the blood of the vertebrate into sex-categories, there remains to be considered a sexual differentiation among the short forms. I have not been able to detect any morphological distinction that could reasonably be attributed to sex. There is a certain amount of variation among the short forms in length and breadth, but it is not marked, and the nuclear features are very uniform. If the short forms do consist of male and female gametes or gametocytes, the differentiation is not expressed morphologically.

Cytology of T. gambiense *in the Blood of the Vertebrate.*

To consider very briefly the finer structure, and the process of division of *T. gambiense*, as studied on blood-films fixed by Schaudinn's corrosive-alcohol solution and stained by Heidenhain's iron-hæmatoxylin method. The body-form of trypanosomes is too well known to require description, and the species in question shows the typical relations. The kinetonucleus is small in size, and shows the usual two aspects, being either rounded or slightly rod-shaped. Close beside the kinetonucleus, at the actual origin of the flagellum, may be seen a small granule, the blepharoplast proper, or basal granule. The specimens derived from monkeys show a finely granular protoplasm, and larger inclusions are very rarely seen. The current view of the trophonucleus of *T. gambiense* is derived from dried Giemsa preparations, and bears only the slenderest relation to the real state of affairs. It is always figured as a large round or oval mass of granular chromatin.

With good illumination and a high-power system, any patient observer can see the nucleus in favourable specimens in the live state. This is, of course, more easily achieved in the larger species; in the trypanosomes of fishes and reptiles, an immersion-lens is not essential. The live picture is in all cases that of a circular, slightly refractile object, lying surrounded by a clear halo. The material fixed by the wet method, and never dried in air at any period during the preparation of the slide, shows conditions very closely resembling the live picture, and utterly different from the version given by the usual Giemsa method. All fixation is probably a choice of error, but it stands to reason that, if a delicate and highly flexible organism occupying three dimensions is swiftly flattened out into two, quite regardless of its attitude at the time, disasters are liable to occur. In dry preparations the nucleus has simply been burst. There is nothing wrong with this method so long as the observer remembers that he has destroyed this important structure, and does not proceed to describe it as though he had respected its integrity. The criterion of fixation must be the relation of the fixed material to the live picture, and, judged by this standard, the wet fixation and passage into some mounting fluid is greatly to be preferred. The criticisms that may be urged against the wet method are slight shrinkage of the protoplasm of the body, and the fact that, as the attitude in three dimensions has been very closely preserved, it is almost impossible in many cases to get an accurate microscopic measurement of the length of the animal. The ordinary methods of measurement supply no means whereby the elevation towards the eye of the observer can be accurately estimated in the case of small objects of irregular shape. Another drawback to the wet method is that all the parasites in a drop of fluid may not adhere to the cover-slip; it is clear that in certain kinds of work this is a very serious disadvantage.

The trophonucleus is of a type very common among flagellates, and is characterised by a large central karyosome, in which almost all the chromatic material is concentrated. This is surrounded by a clear space, which is in turn bounded by a faintly staining membrane or nuclear boundary—the word membrane is convenient, but its use here is not very clearly justified. Very delicate strands radiate out from the karyosome to the membrane; they are not always very clearly visible, but are to be made out in the vast majority of specimens. This condition of the nucleus is extraordinarily constant; practically no variation is found in any of the non-dividing blood-stages, and it is also found to persist through a large portion of the cycle in the fly. An important change does, however, take place in the latter part of the *Glossina* cycle; this will be noticed and discussed in treating of the forms in question (see Plate 1, figs. 1–4).

Division of the Trypanosomes in the Blood of the Vertebrate.

This process resembles very closely the division in the fly cycle, where the details are much clearer, so, to avoid repetition, I shall only draw attention to the most important features. The first sign of division is the doubling of the kinetonucleus. The behaviour of the blepharoplast is not clear, but fig. 6 suggests that it has the centrosomic function to be noted in the division in the fly cycle. The flagellum splits, but never throughout the whole length; it becomes free about two-thirds of the way down, and the flagellum of the daughter individual is usually shorter than that of the parent. The trophonucleus shows one very interesting feature before division. When only the kinetonucleus and the flagellum have as yet begun to show signs of re-duplication, two very well marked dark granules are to be observed on the membrane at opposite poles (figs. 5 and 6); they are generally joined by a fairly thick line to the karyosome. The other strands passing from the karyosome to the membrane persist for a time, but disappear when the division-figure begins to be formed. The two granules just mentioned apparently play the part of centrosomes. I have not been able to trace the exact place of origin of the granules. They seem certainly to be intranuclear, but I am quite unable to say if they arise from the karyosome. The division-figures are shown in figs. 5–8, Plate 1. There is no equatorial plate.

II. Exogenous Cycle in the Fly.

(1) *Conditions Obtaining in the Alimentary Canal of* G. Palpalis *in Relation to the Development of* T. gambiense.

In considering the exogenous cycle, a few points bearing on the habits, etc., of the *Glossina* must be touched upon in passing.

The general anatomy of *Glossina palpalis*, and the structure of the proboscis, and the relations of the salivary glands, are so well known from the excellent work of Minchin, Stuhlmann, and others, that I shall not touch upon them. Following the practice of the Uganda Sleeping Sickness Commission of 1908, I have, in considering the infected flies, divided the portion of the gut which lies between the proventriculus and the origin of the Malpighian tubules, and which constitutes morphologically the mid-gut, derived from the embryonic mesenteron, into three parts, namely, the anterior or thoracic, the middle, and the hinder intestine.

From the dissection of a considerable number of wild tsetses from the Lake-shore, caught only three or four hours previous to examination, it appears that, under natural conditions, the majority of flies certainly do not feed daily. In most cases the whole gut is empty of food-material, except for a small quantity of pale greenish, completely digested fluid in the portion of the gut just anterior to the Malpighian tubules. This condition indicates a fast of at least two to three days, and possibly of a very much longer duration; in captivity, flies will live from nine to twelve days without food if the conditions of moisture are suitable. A large number of experiments of long duration were conducted, in which the cages were starved for two complete days between each feed, but there was nothing abnormal in the number of deaths. The fact that wild flies do not feed at such very close intervals of time as seems to be implied from the usual laboratory treatment of the flies has, as will be seen later, a somewhat important bearing on certain parts of the cycle.

The actual mechanism of feeding calls for attention, in that it affects the course of development. The sucking-stomach or crop leads out of the ventral side of the proventriculus by a long duct; in a full feed, the gut, up to a short distance behind the proventriculus, and the crop are both filled with blood. As digestion proceeds, the blood in the gut diminishes, and that from the crop is gradually passed, first forward along the duct, and then back down the gut. In a smaller feed, the gut may be completely filled up to the proventriculus without blood being taken into the crop at all. Flies are willing to feed before digestion is anything like complete, and it is important to know what occurs when the new blood is ingested. The experiments were made by feeding flies alternately on monkey blood and cock's blood.

The very first feed taken by a fasting *Glossina* (24 to 48 hours old) is usually a full feed, pretty well filling both crop and gut. The next feed, ingested 60 to 72 hours later, may behave in one of the following ways: The new blood may entirely replace all the material of the first feed from both crop and gut alike. In a large number of cases, however, some of the first feed is retained in the crop, and the second feed is taken in on top. In these cases one may also often find that the new blood has replaced all the first feed from the gut. There is thus a mixture in the crop, while the gut is, at the conclusion of the feed, full only of the new blood. In cases where digestion has been slow, the blood in both the crop and the gut may be mixed. Another state of affairs occurs, in which none of the new blood is taken into the crop, although the gut may be filled with the material of the second feed; moreover, in some cases, part of the original blood may be retained in the crop unmixed, and apparently unaltered, for as much as 10 days or more, although frequent feeds have intervened. A case of this kind was also observed in an experiment conducted by Dr. DUKE and Captain FRASER.

The *rôle* of the crop in the economy of the fly appears to be simply that of a store-chamber; no digestion takes place there, and, in flies that have been allowed only one feed, and are thereafter starved entirely, one may find blood, the elements of which are optically unchanged after as much as 12 days. The crop can be completely emptied, and is, indeed, very often found in that state. It is also very frequently found, as is well known, to contain a bubble of gas.

Now, if a newly hatched fly has received trypanosomes with its first feed, if is interesting to see what has been the result of the subsequent clean feed.

This experiment was carried out with small groups of flies in glass jars. They were fed on blood containing numerous parasites; the greatest care was taken to see that all the flies had fed, there being, of course, no difficulty in ascertaining by inspection whether a fly has fed or not. The clean feed was of cock's blood. The results here noted are partially drawn in addition from some jars fed only once, and killed after a suitable interval without a second feed. Further evidence is derived, moreover, from the many early dead flies examined in the course of the general mass of experiments.

(1) The trypanosomes may be digested and disappear from the whole alimentary canal of the *Glossina* during the digestion of the first feed, *i.e.*, during the first 50 to 72 hours, without the intervention of a second feed.

(2) The trypanosomes may survive in the gut in small numbers, but disappear during the early stages of digestion of the new blood.

(3) The trypanosomes may survive and multiply in the gut in the blood retained from the first feed, although a second feed has been taken in on top.

(4) Trypanosomes may survive and develop in the crop for as much as 12 days, provided the crop has never been entirely empty of blood during that period. The gut of these flies may often show no signs of trypanosomes. The trypanosomes in the crop seem unable to survive the emptying of this organ, and there is never a permanent infection in this situation.

(5) The trypanosomes may persist in greater or less numbers in the gut and in the crop of the same fly.

(6) The whole material of the first feed may be displaced from the gut by the second feed, and the trypanosomes still persist. The crop in these cases was either empty or filled with the new blood.

To consider which of the above conditions are likely to lead to an infected fly :—

(1) and (2) may be dismissed at once as negative.

(3) is a state of affairs that leaves the issue doubtful; if the trypanosomes are sufficiently established not to be swept out when the original blood is digested, a positive infection probably results.

(4) seems to suggest a negative result, as the condition of the gut implies that the trypanosomes are being digested and disposed of as they pass out from the crop into the gut.

(5) is a doubtful condition, almost identical with (3), though the chances of a positive result are obviously rather more favourable.

(6) seems to be pretty definitely a condition that will lead to a permanent infection of the fly. Once parasites are fairly well established in the new blood, the rate of multiplication is such that the chance of destruction at the next influx is small, and they have obviously been capable of withstanding the mere force of digestion by itself.

Clearly, two factors come into play here in the question of the establishing of an infection in the *Glossina.* First, the capacity of the trypanosomes to withstand the digestive processes ; (1) and (2) show that this is not a property possessed by all the trypanosomes. The second factor is the clearing out of the trypanosomes by the new feed ; that this is a very potent agent in the production of negative flies is shown by the very much higher percentage of infected flies found up to the 12th day in experiments where the flies had only one feed, and that the infecting one. These experiments were carried out with controls, fed every two or three days in the usual way ; the starved flies lived up to about the 10th or 12th day. Out of 103 starved flies, 22 showed trypanosomes between the 6th and 12th day. Neglecting those flies which showed trypanosomes only in the crop, there were 16 left which showed apparently well-established, though not very numerous, infections in the gut, *i.e.* 15·5 per cent. This is a very remakably high percentage for the ordinary Uganda strain of *T. gambiense.* Under ordinary feeding conditions, the strain used for the starvation experiments just quoted produced 3 per cent. of + flies.

To quote a typical experiment. Jars Nos. 16, 17 and 18, each containing 15 flies, were fed on the same day and within a few minutes of each other on one monkey. Jars Nos. 16 and 17 were fed every third day, and dissected on the 22nd and 28th day of the experiment respectively; only one out of the 30 flies showed trypanosomes, and that was an individual which had died and been dissected on the 2nd day after the infecting feed. Jar No. 18 was starved outright after the infecting feed; between the 9th and 12th days three flies showed trypanosomes in the gut, and three showed trypanosomes in the crop—altogether, 6 out of the total 15 showed trypanosomes. It must, however, be noted that the trypanosomes may disappear entirely from all the flies in a starvation experiment, and fall under the same heading as negative experiments in general.

To obtain therefore a clear idea of the elements that come under observation in the confusing early days of the cycle in the alimentary tract of the fly, it must be borne in mind that there are several conditions which may show trypanosomes persisting.

(I) Mere persistence without multiplication. Here the parasites undergo the usual slight alteration in shape, to be discussed later, and degenerating specimens are also to be seen. This condition is not found after 72–84 hours, and is chiefly to be observed when the trypanosomes were numerous in the infecting blood.

(II) Cases where the trypanosomes persist in small numbers in some part usually of the mid-or hinder intestine and multiply, but are, nevertheless, unable to establish themselves permanently. These are mostly cases where the apparently adverse conditions of the new feed come upon the trypanosomes before they are able to withstand them. In this state dividing and degenerating specimens are to be met with as well as numbers that do not suggest either condition. The individual types will be treated later. Persistence and quite normal development may occur, as has already been stated, in the crop and continue till the 10th or 12th day, after which time I have not observed trypanosomes in this situation until they reappear there occasionally at the end of the cycle. This persistence and development in the crop is very interesting and important for two reasons: first, in that it obviously allows of several chances of infecting the gut, as all the material in the crop, including the trypanosomes, is passed backwards down the alimentary canal. In the second place, it shows that the stimulus to development in the fly is not dependent upon the digestive action of the gut fluid upon the blood, *i.e.* neither the digestive fluid nor its action upon the blood are essential factors in setting off the developmental processes.

(III) The third case found in the early days of the cycle is where the conditions admit of trypanosomes persisting and multiplying with sufficient vigour to establish themselves permanently in the gut. This may occur at any part of the middle or hinder intestine. I have never observed what one could with justice call established trypanosomes in the anterior intestine at an early period. This third condition leads to the production of a positive fly.

Out of a study of all these circumstances it may be deduced that, where no serious attempt at multiplication is made on the part of the trypanosomes, we have an expression of the incapacity of the parasites, and that we are dealing with a negative condition, that is to say, the large majority of the individual flagellates ingested are not in a fit state to carry on the cycle. This condition of the flagellates is, as has been already mentioned, a definite and recurring, though transitory, stage of the endogenous cycle in the vertebrate, and constitutes the negative period during which the short forms are not present in sufficient numbers, or are in an unsuitable state.

The very large number of cases where the attempted multiplication fails to establish an infection may be taken as representing the general inhibiting capacity of the *Glossina*, and appears to be a very fairly constant factor in all experiments dealing with newly hatched flies. The great value from the experimental point of view of the infecting feed being the first feed ingested lies just in the point that only in this way can any sufficient uniformity be ensured in the condition of the alimentary tract of a batch of, for instance, 50 flies. The inhibiting capacity just mentioned is responsible, given a suitable condition in the infecting material, for keeping down the percentage of infected flies. Individual strains of *T. gambiense* vary greatly in the general percentage of infected flies produced, this being due to the greater or lesser vigour of the trypanosomes interacting with the inhibiting forces of the fly. The negative blood periods are just as marked in very vigorous strains as in those of lesser vitality.

(2) *Development of* T. gambiense *in the Gut of* G. palpalis.

To consider now the typical course of the developmental cycle of *T. gambiense* in *Glossina palpalis*. Some general features may be disposed of at once. There does not appear to be an intracellular stage at any part of the cycle, either in the gut or in the salivary glands. Such a development was very carefully searched for, Prof. MINCHIN and Dr. THOMSON's important discovery of this stage in *T. lewisi* naturally adding to the probability of an intracellular phase occurring in other cycles, though *T. lewisi* is obviously a very different type of trypanosome from any of those included in the "*brucei* group."

T. gambiense develops in the lumen of the gut from the very start, and there is no gap or disappearance of the parasites from this situation at any period. The flagellates are never found to attach themselves in any way to the wall of the gut, but do attach themselves to the wall of the salivary gland when established there. In my experience the trypanosomes do not pass through the wall of the gut into the body cavity at any time.

While there is a very definite course of development in the fly before the trypanosomes are ready to infect another vertebrate, nevertheless the duration of this cycle of changes may vary to the extent of a full fortnight. A certain amount of experience with an individual strain will permit an observer to predict with considerable accuracy the state of affairs upon any given day; but it is quite impossible to say with a random strain that any type or condition is typical of a given date. A 10th or 12th day fly of one strain may correspond with a 20th day fly of another. Even a single strain may sometimes vary in regard to time as much as a week or ten days. This must be borne in mind in dealing with the stages of the cycle. Variation in the time-element of development is a well-known biological phenomenon, which has, however, not received much attention in dealing with Protozoa; the present instance is a very marked case. Flies may be infective already on the 17th day, while it is quite common for the cycle not to be complete until after the 30th day.

The earliest case obtained in which the trypanosomes could with certainty be considered as developing, and not merely persisting, is that of a fly of the 2nd day. That is to say, the trypanosomes had been in the gut 36 to 48 hours. A provedly developing infection of so early a date is merely obtained by a fortunate chance, and the evidence in favour of its authenticity is as follows :—Experiment 122 was fed on Monkey 597, an infected individual which was under close observation ; but live and stained films were being examined daily. On the date of the infecting feed, March 5th 1912, 597 showed no trypanosomes in the course of the examination of the live film. Prolonged examination of a stained film, 3 ins. × 1 in , showed very rare parasites. The fly in question (No. 8) died during the night of March 6th–7th, and was dissected at 9 A.M. on the morning of the 7th. It was found to contain partially digested blood and a very considerable number of trypanosomes, which were in an active and lively state, a number far in excess of those found on the stained film taken immediately before the cage was fed It may be mentioned in passing that Experiment 122 afforded two more positive flies when the box was dissected on the 23rd day. Wet fixed films were made as usual from the contents of the gut of the fly, and multiplying individuals were observed. Figs. 9–12 are from this fly. Although special attention was paid to this fly from Experiment 122, as being the earliest obtained, nevertheless the stages correspond very closely with those seen in the relatively large numbers of early positive flies dissected between the 3rd and 6th days. A reference to figs. 9–16 will make this clear.

No very striking or rapid changes occur when the trypanosomes are taken into the gut of the fly. Degenerating forms are present in greater or lesser numbers. The healthy-looking forms show a general but very slight difference from the blood type which is difficult to define in words ; the membrane is slightly narrower ; the kinetonucleus moves somewhat nearer to the trophonucleus, which itself in most specimens increases a little in size. There is no marked division into slender and broad forms ; there is variation in respect to length and breadth, but the categories are not sharply marked off, and the extent of the variation is slight. One point, however, emerges, which is, I think, of real importance, namely, that the dividing individuals at this early stage are all relatively broad forms. Figs. 11 and 16 are typical of the process. A broadening of the individuals about to divide is not characteristic of the development in the gut of the fly as a whole, as a glance at figs 20 and 30–33 will show, the deduction being that in the earliest days it is the broad individuals that have been ingested that are capable of division. This observation tends to confirm the impression received from purely external experimental sources that the broader, shorter forms are responsible for carrying on the cycle in the fly.

The finer details of division will be gone into in the case of the later days, where the cytology of the process is exquisitely clear. One thing is, however, worthy of notice, and that is the relations of the kinetonucleus of the daughter individuals in figs. 11 and 16. In both cases the young individual is still in a crithidial condition, a very transitory state, as it develops almost invariably into a trypanosome before it is set free. (See fig. 12, from 2nd day.) This is only characteristic of the earlier divisions, and (with the exception of a very slender form to be discussed shortly) is the only sign of a crithidial phase to be observed in the gut development. There is, as will be seen hereafter, a most marked crithidial stage in this, as in most tropanosome cycles, but it does not occur in the gut.

As multiplication proceeds there are produced a large variety of shapes and sizes, though the trypanosomes do not seem frequently to surpass the maximum length of the blood type, *i.e.*, 34–35 microns. By the tenth day or thereabouts there are very numerous trypanosomes exhibiting a wide range of form. The characteristic slender form so remarkable in the anterior intestine and proventriculus later in the cycle may begin to appear already, but only in small numbers.

A long thread-like form, such as that drawn in fig. 43, and which comes from a 12th-13th day fly (mid-gut), corresponds apparently to the "male" forms of KLEINE and TAUTE.* There is no evidence in favour of this being a sexual form at all; it has never been found in conjugation, in spite of much searching, either by KLEINE and TAUTE or myself. In dealing with flagellates, there is no support to be found, in any of the well worked-out cycles in which conjugation has undoubtedly been established, for the theory that markedly long slender forms are male gametes. In flagellates, all cases of conjugation involving hologametes (as must obviously be the case here) are practically isogamous.[†] There is no argument by analogy in favour of these long forms, such as fig. 43, being males. The use of analogy is obvious in working with an individual of a very well-known group; no one would hesitate to use this method in working with the Coccidia, for instance, but the analogy, to be sound at all, must be drawn from the group in question.

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* 'Arbeiten aus dem Kaiserlichen Gesundheitsamte,' vol. 31, Part II.

In the case of *T. gambiense*, one must first establish clearly that these forms (fig. 43) do enter into conjugation before they can be held to be male gametes. In the case in point, the evidence is against these forms being gametes. They have never been seen in conjugation with broad or other forms. They are of rare occurrence, and appear in the middle of a period of very active multiplication, which is not a characteristic of gametes generally. I have never seen them earlier than the 12th day, even in the most rapid cycles. Moreover, their nucleus and protoplasm has almost always a pathological appearance, and one is inclined to consider them as degenerating slender forms (compare figs. 40–42).

A great deal of degeneration goes on in a well-infected fly, as is only to be expected when the tremendous numbers of parasites present are considered. These processes are easily recognised in the broader forms, but are much more obscure where the slender types are involved.

The posterior position of the nucleus sometimes seen in these forms seems to be due to the tendency so often shown in degenerating trypanosomes for all the protoplasm and its contents to aggregate at the posterior end (fig. 41). This condition does not seem to me to be really in any way comparable to the usual crithidial phases of trypanosome cycles, where the whole culture passes through a state in which the trophonucleus is definitely posterior to the kinetonucleus, and which is followed by a reassumption of the trypanosome-condition proper (compare the life-cycles of *T. lewisi*, *T. raiæ*, of the trypanosome of perch, goldfish, etc., the cycles of bird-trypanosomes, and the salivary gland phases of *T. gambiense*). Very slender individuals, without any trophonucleus at all, are seen at times; they are rare, and I do not consider that they are in any way normal forms. A limited vitality is preserved for a time in very degenerate specimens, and even in parts of a trypanosome, thus, actively motile flagella, with or without a portion of the membrane attached, are known to persist for some hours before disintegration sets in.

[†] See DOFLEIN's 'Lehrbuch der Protistenkunde,' 1909, for a general account of the Flagellata.

Cytology and Details of Division.

The cytological relations, and the details of division, are very well seen in the gut-forms, and the following account has been drawn from the middle period (10th to 12th day) of the cycle. The body-form, as already mentioned, is subject to the greatest variation. In the multiplicative types (figs. 30-35) the protoplasm is granular and somewhat dense in appearance but does not usually show larger inclusions. The kinetonucleus is larger than in the blood types, and the blepharoplast (or basal granule) of the flagellum is very clearly visible. The trophonucleus contains a large karyosome, apparently very rich in chromatin; the relations are much as in the blood types, only the whole nucleus is generally rather larger, and the nuclear membrane is not quite so clearly marked.

At division, the blepharoplast divides before anything else, and is immediately followed by the first splitting of the flagellum. The two daughter blepharoplasts move to either side of the kinetonucleus, and very clearly and constantly play the part of centrosomes in its division (fig. 30). A centrodesmose is formed between the two blepharoplasts, and division takes place without the formation of an equatorial plate. In well-stained iron-hæmatoxylin preparations, this set of conditions can be seen with the most exquisite clearness and precision. The earliest appearances of division in the trophonucleus are seen in fig. 31, where the two centrosomes, are lying on the slightly drawn-out membrane; they are joined by a centrodesmose, and the first signs of a division of the karyosome are to be observed. The nuclear membrane becomes very faint, but never seems to disappear, so that it seems highly doubtful in how far it is homologous to the nuclear membrane of the Metazoa. The chromatic material draws out along the lengthening centrodesmose into a somewhat irregular spindle, and the karyosomes ultimately re-form without the intervention of an equatorial plate phase. The remains of the spindle-figure, which is sometimes extraordinarily long (it may be much longer than that shown in fig. 34), are absorbed in the protoplasm. The fate of the centrosomes after division is obscure, and I cannot say whether they are incorporated in the daughter-karyosomes, or whether they disappear or lie on the membrane. The division of the protoplasmic body is very characteristic. There is no longitudinal splitting of the patent organism, but the young specimen is really as it were pushed off (or grows off) at the posterior end (fig. 35), and the division is practically transverse. The two organisms never swing out so as to be arranged kinetonucleus to kinetonucleus, as in the majority of trypanosome-divisions. This behaviour is very constant; the young individual is usually somewhat smaller than the parent.

When the slender forms (figs. 36, 37, 39) which constitute the regular proventriculus type come to be developed (this occurs any time after the 10th to 15th day) they arise quite gradually from the broader forms and there is only one point of importance in which they differ from their predecessors (figs. 36 and 37). The body is long and slender, the protoplasm very finely granular and much less dense than in the broader forms, but the salient feature is the trophonucleus, which shows an interesting change. The karyosome has become very much smaller and the membrane has become much more marked and stains deeply, suggesting that it carries some quantity of chromatin: the fine rays can rarely be distinguished, but there are some indications that they nevertheless persist. There seems to be a reduction in the quantity of chromatic material in the whole nucleus, but it is impossible to say whether this is merely in relation to the lesser quantity of protoplasm, or whether it is some sort of a nuclear preparation for the subsequent development in the salivary glands. Division takes place in fairly slender types (fig. 38), but seems to be inhibited in individuals such as those shown in figs. 36 and 37.

The infection as a whole generally develops in the hinder intestine or the posterior part of the middle intestine. I have never found trypanosomes established in the rectum. The infection literally grows forward by sheer force of multiplication till it fills the whole of the middle and hinder intestine and the posterior part of the anterior intestine. The anterior portion of the anterior intestine and the proventriculus show the typical long slender forms (figs. 36 and 37) described in the preceding paragraph, and are not invaded till some time about the middle of the cycle, from the 10th to the 20th day, or thereabouts. There seems to be some difficulty in the trypanosomes reaching the preventriculus, and, once arrived there, they maintain themselves in that situation only so long as the fly is not exposed to too long a fast. If a fly has digested its meal and there is any considerable interval before the next is obtained, the trypanosomes ebb backwards to the posterior part of the anterior intestine, and only gradually recapture their position again If, however, the new blood is taken in while the trypanosomes are still in the proventriculus, they retain their position and are apparently little affected by the influx of fresh blood.

These conditions can easily be observed from a series of simple experiments with boxes of the right age, in which the feedings have been suitably spaced and their relation to the state of the gut of the positive flies carefully observed.

This inability on the part of the parasites to maintain themselves in the proventriculus during a fast becomes of some importance in the cycle when the habits of the wild flies in regard to feeding, referred to earlier in this paper, are remembered. The majority of wild flies are obviously exposed to fasts of considerable duration, and it is this circumstance, combined with the peculiarity of the trypanosomes just mentioned, that seems to necessitate some seat of attachment for the trypanosomes anterior to the proventriculus in all cycles developing in *Glossina palpalis*. In *T. gambiense* the flagellates invade the salivary glands; in *T. pecorum* and *T. nanum*, as will be shown later, they attach themselves in the proboscis after having gone through a gut-development not unlike that of *T. gambiense*; in *T. vivax* and *T. uniforme* the whole cycle takes place in the proboscis. There is at present, as far as I am aware, no case of a completely investigated gut form carried by *Glossina palpalis* being transmitted directly from the alimentary canal without some definite secondary focus occurring anterior to the proventriculus.

In very numerous infections the trypanosomes sometimes overflow from the proventriculus into the crop, and may be found there in considerable numbers; they are typical slender proventricular forms. When injected into a clean monkey they are incapable of producing infection.

The gut-development may be said to culminate in the slender proventricular type. The hinder and middle regions of the intestine right up to the death of the fly present a medley of multiplying forms differing little from those produced in the first 12 days. There does not appear to be much, if any, multiplication among the very slender individuals, and the hinder and middle intestine seems to serve as a reservoir from which the more slender types are being constantly drawn.

In many trypanosome cycles, notably those of fish and reptiles, this slender form is the last phase of the development in the invertebrate, but this is not the case in *T. gambiense.* An essential development differing very markedly from that carried on in the gut still remains to be gone through in the salivary glands. Before, however, leaving the gut cycle it is necessary to consider two very important points: (*a*) The meaning of certain curious multiple and non-flagellate forms found in the gut from about the 5th to the 12th day; and (*b*) the question as to whether conjugation has taken place at any period in the gut cycle just sketched.

(*a*) In live preparations of the gut there may occasionally be seen confused motile forms with several flagella; others are somewhat amœboid, and the flagella are more or less adherent to the protoplasm; others have no flagella at all, and are quiescent; and still others are obviously wriggling masses of half-fused trypanosomes. Figs. 21–24 give some of these forms as they appear in stained preparations. They always lie right up against the peritrophic membrane. Now, there is little doubt from the fact that these forms can be seen in live films to be caused by the fusion of soft, rather unhealthy-looking trypanosomes under the unfavourable condition obtaining on the slide, that many of these appearances are cases of degeneration. Nevertheless, this is no proof that all the multiple individuals are degeneration products, to be dismissed without further consideration. I have not been able to get any evidence that the multiple forms play any progressive or essential part whatsoever in the cycle. They must, however, always be borne in mind, as these appearances may afford an unfortunate mask, obscuring the process of conjugation. Sometimes in live films from the early days in the fly-cycle, and occasionally also on films from the monkey when these were prepared with a little water or with a 0·50 per cent. salt solution, trypanosomes were observed to come together from opposite sides in pairs so as to overlap about one-third of the body length as in fig. 39A, which is from a freehand sketch of live individuals. The junction seems superficially to be extraordinarily close, suggesting fusion even under a high-power lens, nevertheless the specimens have always come apart again, the longest period of apposition observed being 35 minutes. Usually the individuals are quite similar to one another; on one occasion, however, in the case of a 3rd day fly, they showed a slight amount of differentiation, one being more slender than the other. The "male" type (fig. 43), discussed earlier in the paper, was not involved in any of these cases, in all of which the trypanosomes were of the blood type.

This is the sum of the direct evidence from live observation that I have been able to obtain in the course of more than a year's search, and it is obviously inadequate. The trypanosomes do not live long enough under the cover-slip to give satisfactory opportunities for this kind of observation. The fact that the divisions are so very characteristic and so constant in type would admit of valuable deductions being made from the stained material, were it not that the multiple degeneration and involution forms obviously rob these of all their value. It must also be considered whether conjugation might not take place in the salivary gland. Direct evidence there is none, and live observations are even more hopeless here, as the trypanosomes from the glands are extraordinarily sensitive to the unfavourable conditions of the slide. As will shortly be seen, there are important data for considering the salivary gland cycle as the really essential part of the whole development, and it is for that reason that one is inclined to entertain the idea that the sexual process might occur in this situation.

(*b*) Theoretically there is a good deal of evidence in favour of conjugation or some equivalent process occurring during the cycle in the transmitting host. The passage through the *Glossina* as a whole seems certainly to have the same biological significance. There is clear evidence in certain cases observed here at Mpumu—the case of Monkey 199 was cited in this connection in an earlier paper—that peculiarities acquired by a strain during its sojourn in a particular host are eliminated by passage through the fly, whereas these idiosyncrasies are retained when the strain is passed directly by injection. There can be no doubt that from a consideration of the life-history as a whole the part passed in the fly plays a definite and essential *rôle* in maintaining the integrity of the species, quite apart from its being a convenient method of transmission.

(3) *Stages of* T. gambiense *in the Salivary Glands.*

The penetration of the trypanosomes into the salivary glands occurs quite clearly from the hypopharynx, and the successive stages of the process can be seen very well in the live state, in careful dissections of the glands at the appropriate periods. An individual trypanosome cannot, of course, be watched through the process, but the study of a good number of flies leaves no shadow of doubt as to the sequence of development. The easiest way to get at the thin duct of the glands, which is the place where the first stages are to be found, is to starve the fly for full two days, then to cut off the posterior tip of the abdomen, snipping off a slightly larger portion than is usual for ordinary dissection, and to pull out the gut without breaking it off. This minimises the chance contamination of the gland with the contents of the gut. If the glands do not appear, stroke the abdomen gently with the flat of the needle until they do; when the tip of the gland emerges, a single rather slight stroke of the needle will bring it out right up to the narrow duct, and the same for the other side. The glands are transferred to a clean slide with a drop of normal salt solution or monkey serum; they are transferred again to a clean slide and examined. The pulling out without breaking the gland is an easily acquired knack and should be done under a low-power dissecting microscope.

The slender trypanosomes pass up into the hypopharynx from the proventriculus. The period at which this happens is in direct correlation with the vigour and number of the trypanosomes in the fly, and early infectivity is generally a character found in a strain which produces many positive flies. These slender trypanosomes may in rare cases be seen lying, in small numbers, free in the hypopharynx of flies whose salivary glands are not yet infected. They then come back along the narrow duct of the salivary glands, and can be seen there as free-swimming slender creatures.

The gland, as is well known, is composed of (1) a narrow tubular part, which leads back to (2) a slightly broader cellular part, which in turn leads to (3) the glandular part, where the full width of the organ is attained.

The trypanosomes settle down and attach themselves in the second part of the gland, or at the entrance to the third part, the rest of the gland being quite free from trypanosomes. At first they are slender forms, which sway forwards and backwards, attached by their flagellum to the wall of the gland (figs. 44 and 45). Later on they become broad, round-ended, crithidial flagellates. They multiply, and gradually, what with divisions and fresh arrivals, finally fill up large portions of the gland with flagellates in all stages, from tadpole-shaped crithidias, attached by their flagellum, to free-swimming trypanosomes, closely resembling the blood type (figs. 44-60). These trypanosomes are usually below the length, but not below the minimum measurement, of the forms occurring in the blood. Division takes place among the trypaniform, as well as among the crithidial, parasites (figs. 49, 56 and 57). The cytology of the gland forms calls for no special notice, except that there seems to be an increase of chromatin within the nucleus, and there is a tendency for the karyosome to increase once more in size. The occurrence of marked crithidial, and in some cases almost herpetomonad, forms is very striking; they constitute a large proportion of all the gland types. Degenerating individuals are found in all stained films, but the great sensitiveness of the trypanosomes to the process of investigation leads one to imagine that some of these at least are due to manipulation. While the crithidial forms are mostly attached they may nevertheless be found free also; it seems probable, however, that they attach themselves again.

The fly is, apparently already capable of producing an infection in the vertebrate when only the proximal part of the gland close to the duct shows parasites. The *Glossina* seems to become infective about 2 to 5 days after the trypanosomes invade the gland, but, as one cannot both dissect and subsequently observe an individual fly, this time is naturally only an estimate made from the consideration of a number of only more or less similar cases. It appears to be clear, beyond question, that only when the salivary gland shows trypanosomes is the fly infective. Early positive transmissions are always accompanied by rapid virulent infections in the fly and an early invasion of the glands. In one case a salivary gland was found infected on the 12th day, but this is quite exceptional—on the 16th day infected glands are found in rare cases, and after the 20th day they become increasingly frequent.

Cycles of *T. nanum* and *T. pecorum* in *G. palpalis*.

It is interesting briefly to compare the general course of development in *T. nanum* and *T. pecorum* with that of *T. gambiense*.

The development of *T. nanum* in *Glossina palpalis* has many features that closely resemble the conditions in *T. gambiense*. The infection starts in the hinder intestine, and by the 10th day (no material was obtained from the very earliest days) numerous trypanosomes are to be found in the hinder and middle intestine. They show a considerable range of form, and have nuclear relations practically identical with those of *T. gambiense* (figs. 61–64). The method of division corresponds in such minute detail with that described in figs. 30–35 for *T. gambiense* that it is needless to recapitulate the description. Slender forms begin to be produced from the 10th to the 14th day of the cycle onwards, and the proventriculus comes to be invaded about the 20th day. The proventricular forms, while generally slender, do not show such a uniformity of type, nor are they so thread-like, as in the case of *T. gambiense*. Moreover, the characteristic change in the nucleus of *T. gambiense* shown in figs. 36 and 37 never takes place at all in the gut forms of *T. nanum*. About the 25th day trypanosomes begin to be found in the proboscis attached to the labrum, often lying in clusters. They assume the crithidial condition, many of them being extraordinarily long and slender (figs. 65–68). No reliable information was obtained concerning the nuclear detail of these types (the drawings are all from dried Giemsa preparations), as it was found that the trypanosomes could not be made to adhere to the cover-slip in the wet preparations, except in very rare instances. Besides the very long crithidial types, shorter forms, such as that shown in fig. 68, were observed, also crithidial in type. In live preparations, free trypaniform organisms were observed, sometimes in the hypopharynx and sometimes in the labrum.

As in *T. gambiense*, the gut forms do not attach themselves to the wall of the alimentary canal, nor are crithidial forms ever seen in the gut cycle. The salivary glands are never invaded in the case of *T. nanum*, the proboscis-infection apparently playing the *rôle* of the gland phases in the cycle of *T. gambiense*.

Trypanosoma pecorum develops in *G. palpalis*, but the cycle is so extraordinarily slow, and the transmission so difficult to effect, that it appears clear from Dr. H. L. Duke's experiments that this fly is at most only a facultative intermediate host for *T. pecorum*. For this very reason the conditions of the cycle have some points of special interest, as will be seen later. The course of the development in the gut resembles that of *T. nanum* and *T. gambiense*; multiplication occurs, and numerous types, varying in length and breadth, are formed (figs. 69–71). The flagellates are more massive and larger than in either of the two forms just mentioned. The slender forms are produced as usual after the broad individuals, and are found in association with them; the long forms are extraordinarily attenuated, and the nuclear change remarked upon in the corresponding stages in *T. gambiense* occurs also here (fig. 72). The first invasion of the proventriculus was observed upon the 45th day of the cycle, and no proboscis-infection was found before the 76th day. The proboscis-infections were generally slight, and the usual difficulties were experienced in getting preparations from the situation. Figs. 73 and 74 show two individuals from a wet preparation, fixed in Schaudinn's fluid, and the nuclear relations recall those of the gland-stages of *T. gambiense*. The salivary glands never become infected.

GENERAL CONSIDERATIONS AND CONCLUSIONS.

The most remarkable feature of the cycle of *T. gambiense* is the curious double development, first in the gut, culminating in the long slender form, and then in the salivary glands, where the crithidial stage so typical of all trypanosome cycles is so marked a feature. It seems difficult to escape the deduction that the gut-development is a somewhat indifferent multiplication, a mechanical device to enable the trypanosomes to establish themselves in sufficient numbers in contact with the salivary fluid, which in the fly seems alone capable of stimulating the trypanosomes to that apparently essential reversion to the crithidial type. In *T. pecorum* and *T. nanum* the gut-development follows in all essentials the scheme of *T. gambiense*, and here also, as already mentioned, the proventricular forms are not infective. While the double nature of the cycle is perhaps more obvious in *T. gambiense* than in *T. pecorum* or *T. nanum*, the cases are, nevertheless, close parallels. The parasitism in the case of *T. gambiense* is obviously of a more complete type, as the invasion of the salivary glands lends greater permanence and stability to the apparatus of infection than the more or less intermittent infection of the proboscis found in *T. pecorum* and *T. nanum*.

In accordance with the foregoing, it may be said that the gut-conditions of *G. palpalis* do not permit of the complete and essential development of any of the group of trypanosomes mentioned in this paper. So long as the parasites are established in the gut only, their presence is indifferent and negligible from the point of view of infection. This is very striking in those rather rare cases where flies infected with *T. gambiense* may show considerable numbers of flagellates as late as the 56th day without the salivary glands being infected. Such flies are invariably harmless. *T. pecorum* may also be considered in this connection; the culture in the gut persists for 70 days in a harmless state, only becoming infective when the parasites do at length succeed in establishing themselves in the proboscis, and going through the crithidial development.

In conclusion, it may be remarked that, if a sexual process is a necessary feature of the cycle passed in the transmitting host, it will probably occur at that stage which seems absolutely essential to the production of a trypanosome viable in the blood of the vertebrate, namely, the crithidial phase in the salivary glands or proboscis.

It is obvious that much of the foregoing work has been simply to carry somewhat further the researches of MINCHIN, ROUBAUD, BRUCE, and KLEINE, more especially of the last two workers. There are no serious discrepancies between the cycle in the fly sketched by BRUCE, HAMERTON, and BATEMAN, and that described above, except that I consider, as has already been said, the fly-history to be in reality a double development. In many points my work is also in agreement with that of KLEINE and

Taute, except that I do not consider that the "male" forms described by him play any important part in the cycle.

A further discrepancy consists in the view held by the German workers at the time of writing their paper, in regard to the salivary gland phases being a non-essential part of the cycle. My interpretation of the endogenous cycle in the blood of the vertebrate is at present, as far as I am aware, unconfirmed by other workers, largely, I imagine, owing to the fact that the interest has been concentrated for some time past on the appearances in the fly rather than on those in the vertebrate.

Summary.

1. *T. gambiense* undergoes an endogenous cycle of development in the vertebrate in the circulating blood. This cycle is of irregular duration and is repeated many times in the course of the disease,

2. The short forms may be regarded as the adult blood types; the intermediate types are growth-forms, proceeding to the long individuals, which are those about to divide, The products of division give rise, directly or indirectly, to the adult forms. The adult forms appear to be alone responsible for carrying on the cycle in the transmitting host.

3. The multiplication occurs in the circulating blood.

4. Multiplication of the parasites was never found within the cells of the liver, spleen, or lungs in monkeys.

5. Rounded non-flagellate types were found on one occasion in the lung, liver, and spleen of a virulently infected monkey. They appear for the most part to be destined to destruction, but it is not excluded that they may survive in small numbers as latent forms.

6. In the fly the trypanosomes are first established in the posterior part of the mid-gut. Multiplication occurs, and trypanosomes of very varying sizes are produced.

7. From the 10th or 12th day onwards slender long trypanosomes are to be found in increasing numbers. These finally move forward to the proventriculus and are the dominant, though not the only, type seen there. The proventriculus becomes infected as a rule between the 12th and 20th days.

8. The salivary glands become infected by the slender proventricular

types. They reach the salivary glands by way of the hypopharynx; arrived in the gland, they become attached to the wall and assume the crithidial condition. Multiplication occurs, and finally small trypanosomes are produced, closely resembling the blood type. The passage through the crithidial stage is the characteristic of the salivary development and the trypanosome forms just mentioned are derived from the crithidial types. The development in the salivary gland takes from two to five days before the forms are infective.

9. The fly is never infective until the glands are invaded. Trypanosomes from the proventriculus when injected into a monkey never produce infection. Trypanosomes may be found in the salivary glands as early as the 16th day of the cycle. An early infection of the salivary glands is always preceded by a very virulent and rapid gut infection.

10. The trypanosomes are never attached to the wall of the alimentary canal, and there is no intracellular multiplication in the gut cycle. A crithidial stage does not occur in the gut cycle. The trypanosomes are never found in the body cavity nor are they ever established in the rectum.

11. Conjugation has not been observed, nevertheless the fly cycle as a whole has the biological significance of conjugation.

12. The cycles of *T. nanum* and *T. pecorum* agree with that of *T. gambiense* in showing a gut development without a crithidial phase. The crithidial phase occurs in the proboscis, where the flagellates attach themselves. The salivary glands are never infected in the case of *T. nanum* and *T. pecorum*.

DESCRIPTION OF PLATES I TO VI.

The figures are numbered consecutively. They are all drawn at an approximate magnification of 3,000 diameters, with the aid of the drawing apparatus of Abbé.

Trypanosoma gambiense.

Figs. 1–4.—Trypanosomes from blood of monkey.
,, 5–8.—Division of blood-types.
,, 9–10.—Trypanosomes in the middle intestine of *Glossina*, 36–48 hours after ingestion.

Figs. 11–12.—Division in the middle intestine, 36–48 hours after ingestion.
,, 13–15.—Forms from the hinder intestine, 3rd to 4th day of cycle.
,, 16.—Division in the hinder intestine, 3rd to 4th day.
,, 17–19.—Forms from the middle intestine, 5th day.
,, 20.—Division from 5th day.
,, 21–23.—Multiple forms from the 6th day of cycle ; 21 is obviously a degenerative appearance.
,, 24.—Involution form from 6th day.
,, 25–29.—Miscellaneous gut forms from the 12th to 20th day.
,, 30–35.—Details of division.
,, 36–37.—Slender proventricular types, final form of the gut development.
,, 38–39.—Slender forms in middle intestine.
,, 39A.—Sketch of live trypanosomes, from slide, from the middle intestine on the 3rd day.
,, 39B.—Non-flagellate form from liver smear of Monkey 653.
,, 40–45.—Early slender forms degenerating in the middle intestine ; 43 seems to correspond to the "male" type of KLEINE.
,, 44–45.—Specimens newly arrived in the salivary gland.
,, 46–55.—Typical salivary gland forms ; note the crithidial condition.
,, 56–57.—Division figures in the salivary gland.
,, 58–60.—Final trypanosome types in the salivary glands, probably the infecting form.

T. nanum.

Figs. 61–63.—Gut types from 14th day.
,, 64.—From the proventriculus, 21st to 25th day.
,, 65–68.—From the proboscis, crithidial forms ; note length of 65, stained by Giemsa, dry method.

T. pecorum.

Figs. 69–71.—Gut forms, 43rd to 49th day.
,, 72.—Proventricular type, 104th day.
,, 73–74.—Proboscis forms, crithidial types, 76th day.

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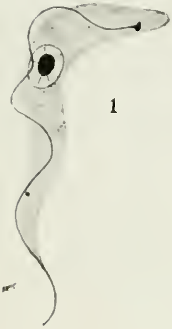
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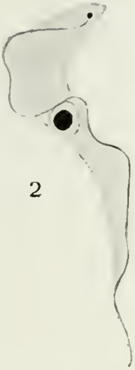
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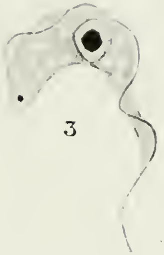
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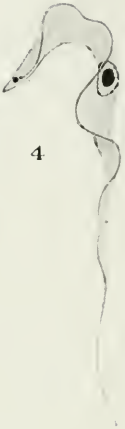
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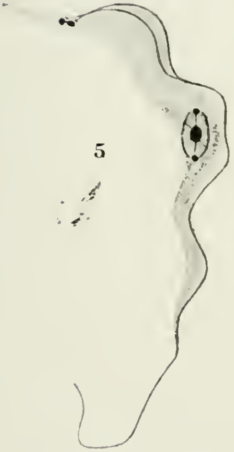
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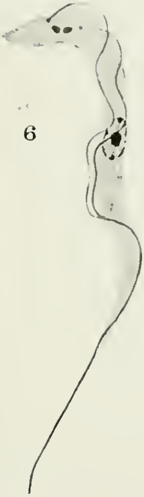
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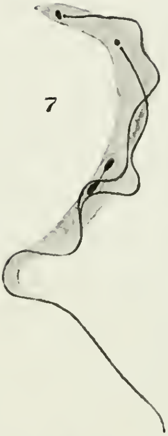
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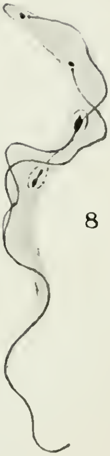
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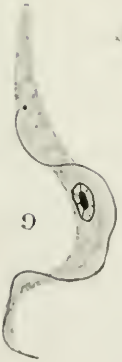
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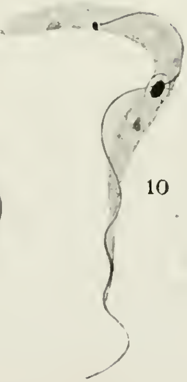
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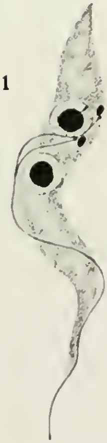
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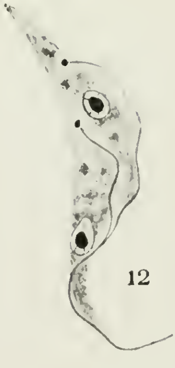
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Plate 2.

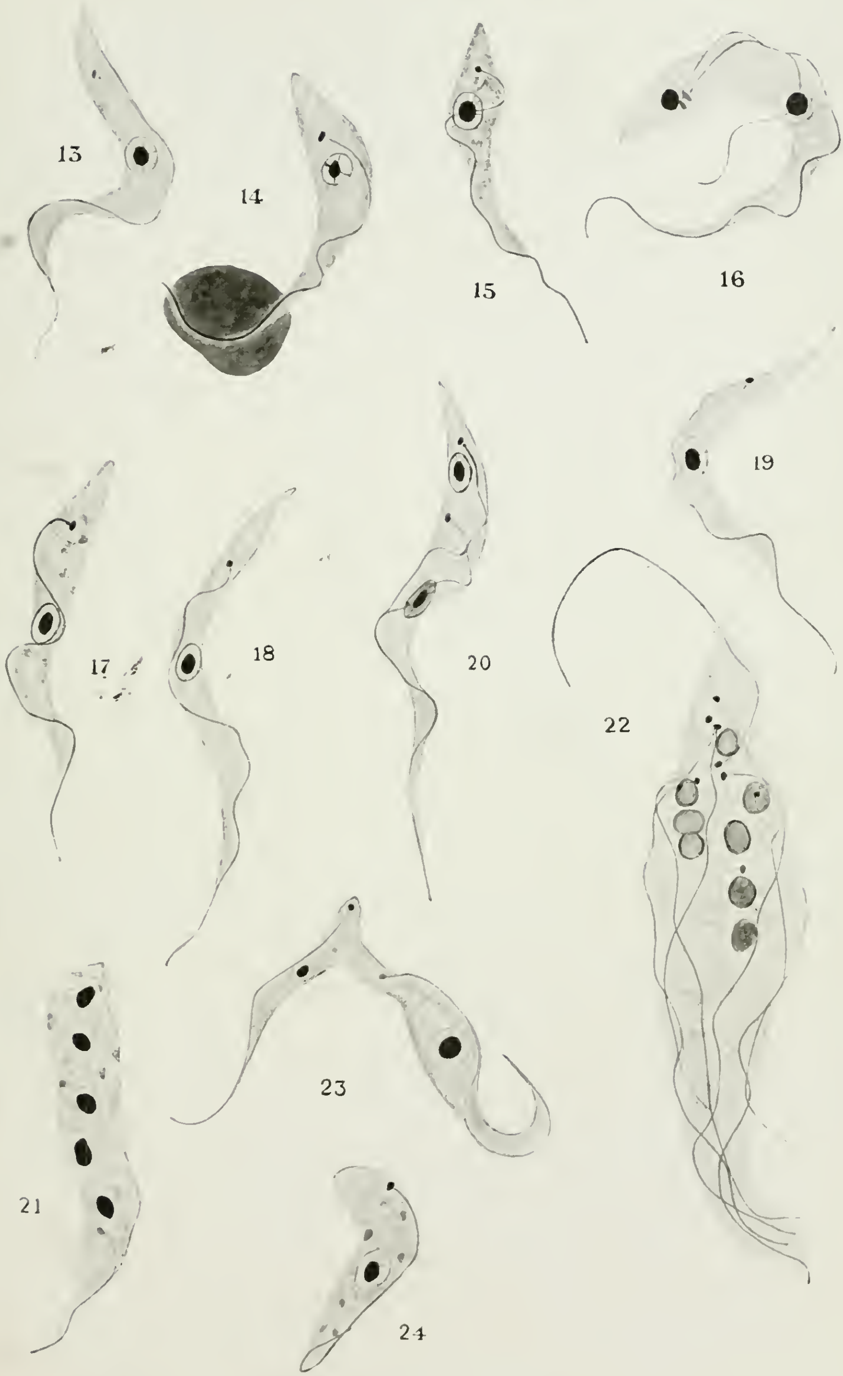

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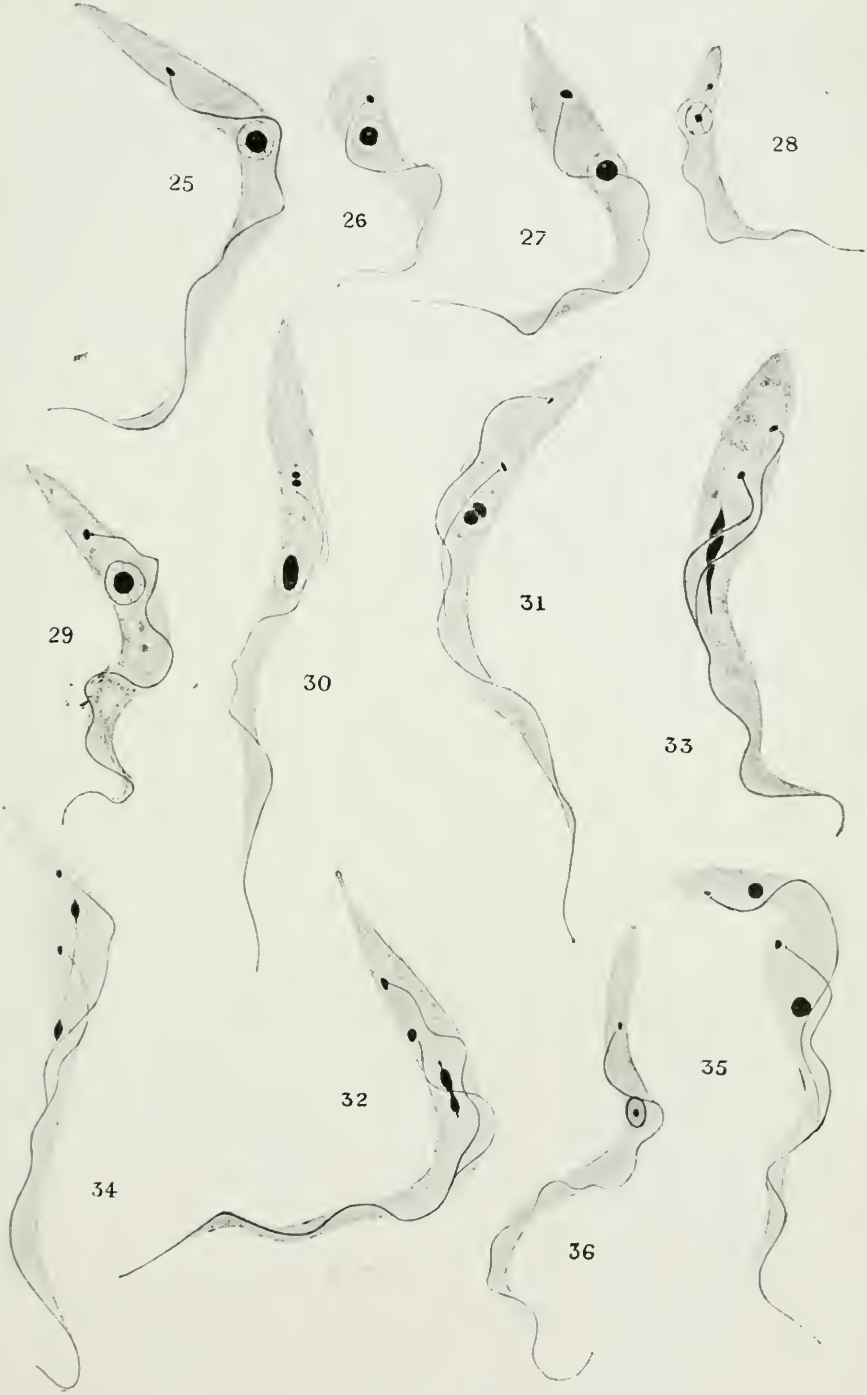
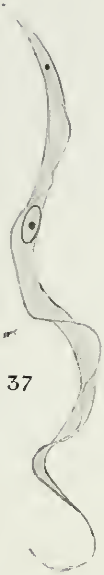
Plate 3.

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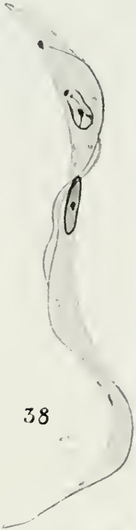
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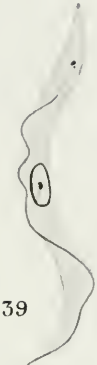
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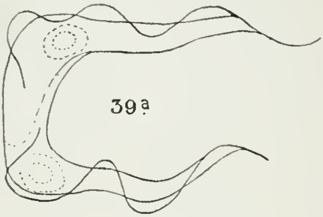
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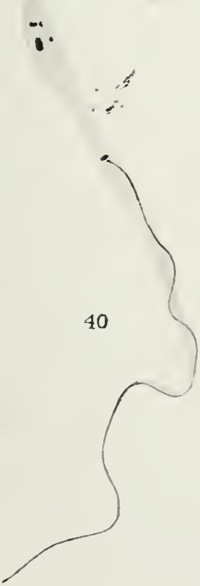
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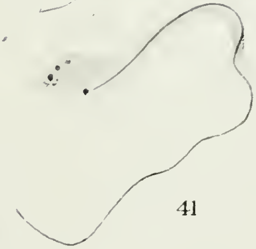
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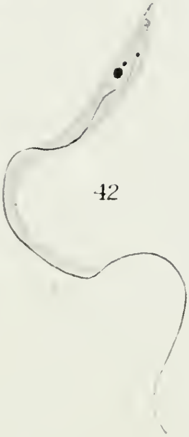
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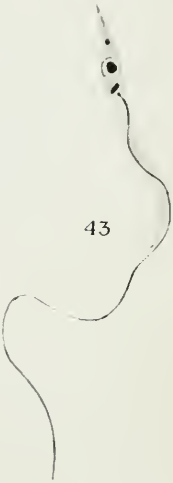
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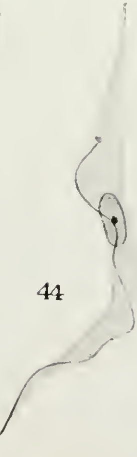
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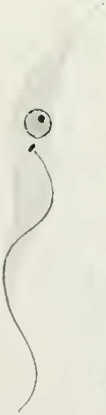
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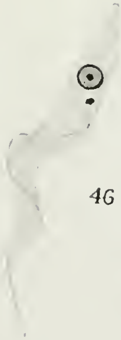
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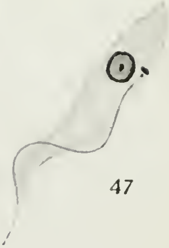
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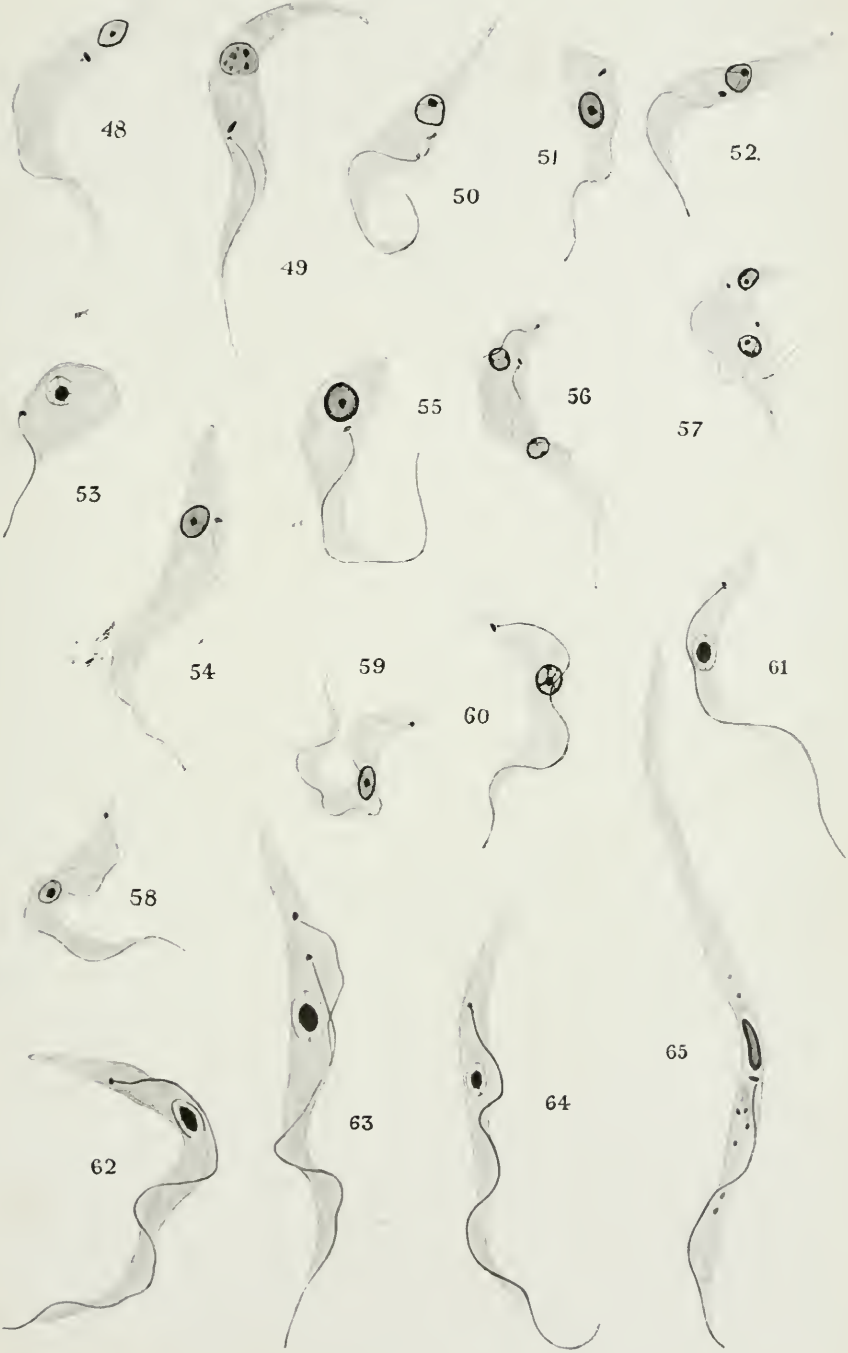
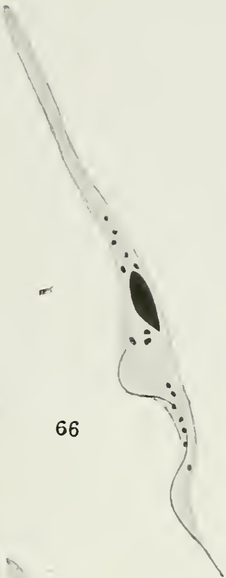
ROBERTSON. *Plate 5.*





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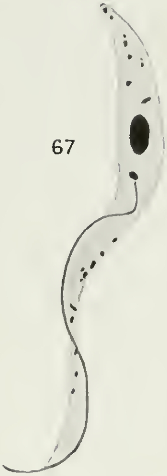

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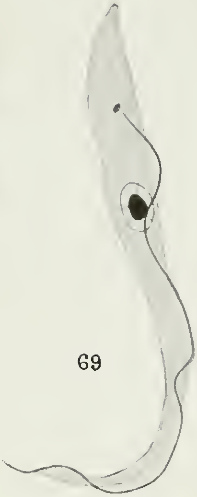

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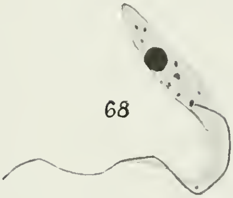

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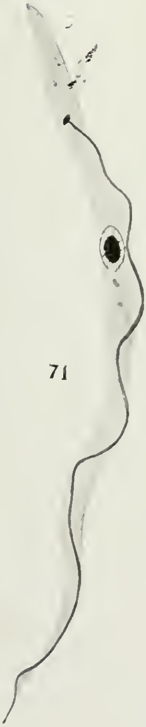

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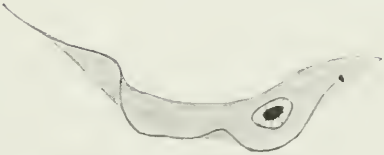

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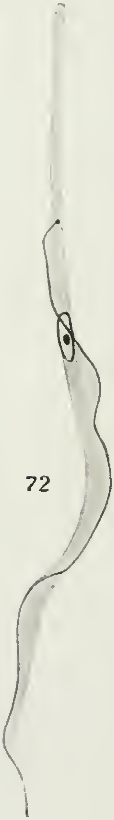

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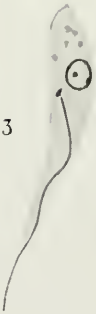

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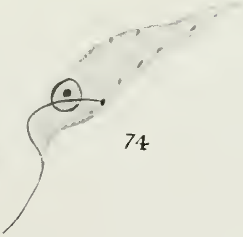

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N° 3

AN INTRACELLULAR PARASITE DEVELOPING INTO SPIROCHAETES,

Found by the Jelly Method of *in vitro* Staining in Syphilitic Lesions and in the Circulating Blood during the Secondary Stages of the Disease.

BY EDWARD HALFORD ROSS,

(The John Howard McFadden Researches) The Lister Institute.

1.—AN INTRACELLULAR PARASITE OF GUINEA-PIGS DEVELOPING INTO SPIROCHAETES.

BY the jelly method of *in vitro* staining,[18] certain peculiar inclusions found embedded within the mononuclear leucocytes of the blood of guinea-pigs and known as Kurloff's bodies were shown to be parasites.[10] These same bodies, if examined by the usual methods involving drying and fixation, merely appear as opaque semi-stained "splodges," and therefore can be easily passed over as artefacts. Hence their true nature was not recognised until the jelly method was employed. The method enabled the development of this parasite, which was named *Lymphocytozoon cobayae*, to be demonstrated. It showed how the chromatin within the inclusion becomes formed into spirochaete-like bodies, and how, after the inclusion has burst, the spirochaetes swim freely in the blood. They can then be seen readily by the dark-ground illuminator as motile filaments swimming in the blood of the guinea-pigs. In addition to this, amoeboid forms were also observed. In the description of this parasite, the suggestion that these spirochaete forms might be microgametes (male element or spermatozoon) was quoted[7]; and the observations were made that the parasite produces anaemia, and that many of the infected guinea-pigs—a large percentage of guinea-pigs bought in England are infected—exhibited single or multiple white patches or tumours within the substance of the liver and spleen.

2.—THE HISTOLOGY OF THE WHITE LIVER TUMOURS OF GUINEA-PIGS.

Microscopic sections of these gumma-like tumours of the liver and spleen of guinea-pigs were then furnished me,[2] and these showed that the tumours contained masses of mononuclear cells, the majority of which exhibited the cell inclusions known as Kurloff's bodies.

3.—THE HISTORY OF THE PARASITE OF GUINEA-PIGS.

Kurloff[3] thought that the cell-inclusions were vacuoles; another[4] suggested "Secretstoffe"; while again others[5,6] claimed their parasitic nature. More recently it has been maintained[8] that they belong to the Chlamydozoa or Cytoryctes. The supposition of their parasitic nature, however, led to conjectures as to the mode of transmission, and food, ectoparasites, and contact have been suspected. But I have already mentioned that by means of the jelly method these Kurloff bodies have now been proved to be living parasites; and observations point to the transmission of these parasites from guinea-pig to guinea-pig being effected at coitus, and also transferred from mother to offspring.†

4.—AN INTRACELLULAR PARASITE OF EARTHWORMS DEVELOPING INTO SPIROCHAETES.

J. W. Cropper[9] found similar cell inclusions within the fixed mononuclear cells of the vesiculae seminales of earthworms; these very closely resemble in appearance the parasites found in the blood of guinea-pigs. By the jelly method Cropper demonstrated the development of these earthworms' parasites through precisely similar stages to those undergone by Kurloff's bodies into free swimming spirochaetes (seen readily by the dark-ground illuminator) which are to be found in the seminal fluid of the infected earthworms.

5.—AN INTRACELLULAR PARASITE DEVELOPING INTO SPIROCHAETES FOUND IN HUMAN SYPHILIS.

A review of the foregoing facts led to the examination of the sores of syphilitic patients by the jelly method, and cell inclusions similar to Kurloff's bodies were found at once. The first case examined suffered from a non-ulcerated Hunterian chancre, which was pricked with a needle and the blood placed upon a jelly and examined. Several of the mononuclear leucocytes exhibited distinct cell inclusions, each one of which was embedded within the cell cytoplasm itself, surrounded by a cell wall, and having a distinctly staining chromatin mass within its interior. In the same specimen round or pear-shaped bodies were seen lying free between the blood corpuscles; these were similar in shape, size, staining reactions, presence of chromatin nucleus, cytoplasm, granules, as those seen included within the cells.

Since then 143 cases of primary and secondary syphilis have been examined by this method, and the intracellular and extracellular bodies have been found in every case. They have been observed in the chancres, in the "hard shotty" non-suppurating glands removed aseptically, in the blood from the maculae and condylomata, in the ulcerated tonsils, and in the peripheral (finger) blood. And in consequence of the number which have been observed, their resemblance to the parasites of guinea-pigs and earthworms described above, and because of their characteristic appearance when seen stained by the jelly method, their parasitic nature is evident; and now various phases of their development have been demonstrated, and the mode of the production of the spirochaete stage seen. The interpretation which I place upon the phases of the development observed in this parasite are as follows: The small extracellular round or pear-shaped bodies, numerous in the chancres and glands (homologous to the free, amoeboid forms of *Lymphocytozoon cobayae*), become included within the cytoplasm of the leucocytes, epithelial and other cells. Here they encyst themselves in a cell wall, grow, and their chromatin undergoes division and subdivision. These included bodies are very frequently seen in the depths of the chancres, glands, and sores. They are found multiple or single embedded within the cytoplasm of the large mononuclear cells; and as the parasites grow, the nucleus of the cell becomes distorted and squeezed. The chromatin within the parasite stains (on the jelly to be described) purple – each chromatin mass having in its centre a well marked very deeply staining dot. There may be one chromatin mass within the inclusion, or there may be as many as twelve, according to the size and stage of the development of the parasite. Each of these chromatin masses is either round or pear-shaped, and some of them may be seen apparently in the act of subdivision. In the full-grown parasite commonly found included within the large mononuclear cells squeezed from the deeper parts of the chancres, or found in the glands removed by operation, each individual chromatin mass will be seen to resemble the free, round or pear-shaped forms already described. In fact, the cell inclusion can be made to burst on the jelly by pressure on the cover-glass, and the contained young parasites extruded.

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† The parasite has just been found by Cropper in the peritoneal cavity of newly born guinea-pigs.

But there is another phase to be recorded. In some instances the chromatin mass within the cell inclusion gives rise, while still within its host cell and when it has reached full size, to several minute purple-staining, curled and twisted spirochaete-like tails (many of them closely resembling *Spirochaeta pallida*) similar to the spirochaete-like bodies seen in the inclusions found with the cells of guinea-pigs and earthworms. If these inclusions are burst the spirochaetes can be extruded.

All the forms here described have been seen in the chancres, glands, and sores of syphilitic patients, and also, but less frequently, as might have been expected, in the circulating blood. They have been seen apart from syphilis, although a great many controls of blood and tissues have been examined on the jellies.

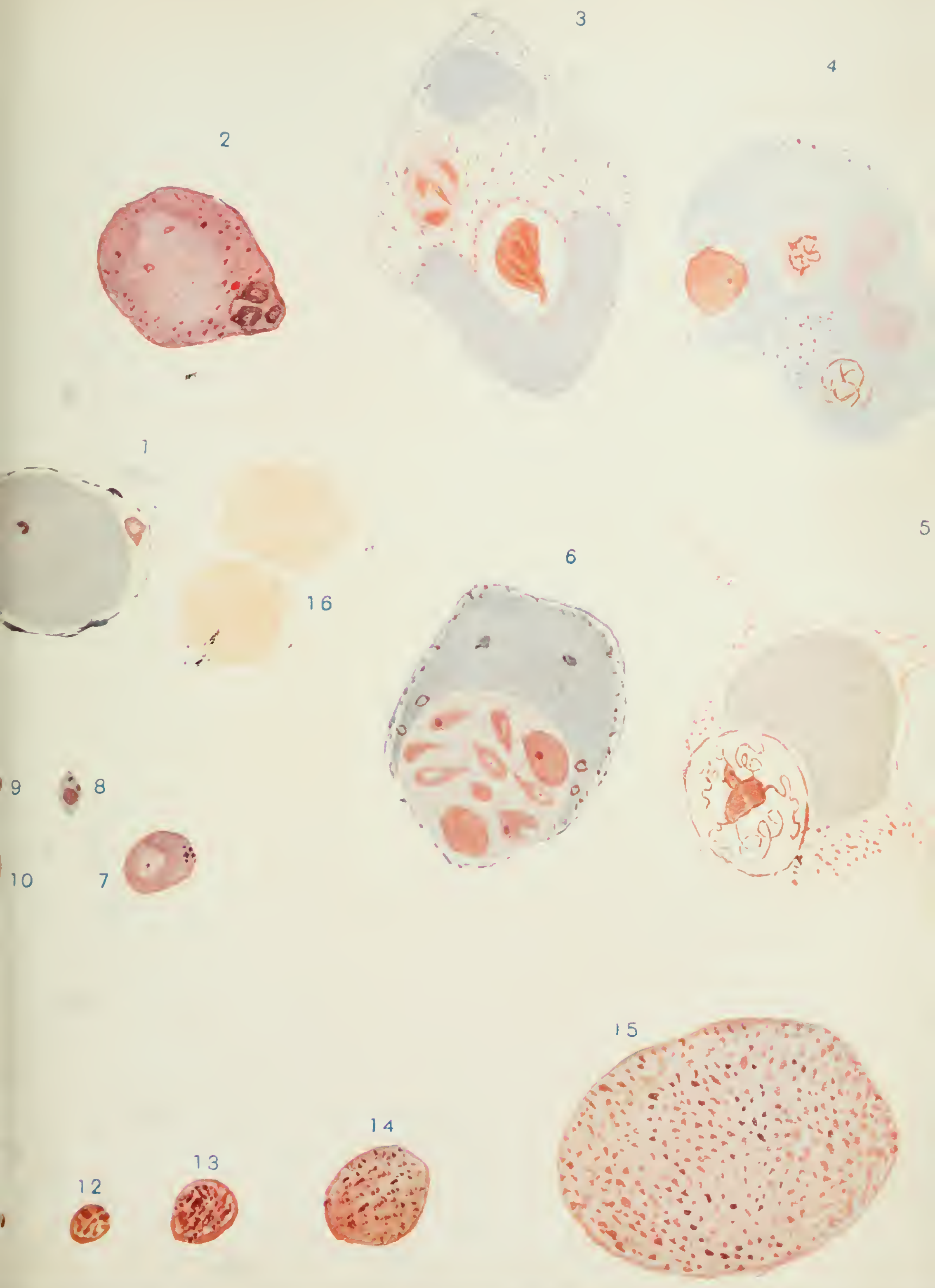
6.—HOMOLOGIES BETWEEN THE HUMAN PARASITES FOUND AND THE INTRACELLULAR PARASITES OF GUINEA-PIGS AND EARTHWORMS.

The parasitic cell inclusion of the guinea-pig has been shown to develop into spirochaetes; the parasitic cell inclusion of the earthworms has been shown to develop into spirochaetes; the parasitic cell inclusion in human syphilis has been seen to develop into spirochaetes, and the presence of spirochaetes in syphilis is well known; but the human parasite has been found to develop into free, round, or pear-shaped bodies as well. It is true that free amoeboid forms were also found in the blood of guinea-pigs, and these have been mentioned by Hunter,[6] but their parallel development in the guinea-pig had not been observed. When these pear-shaped bodies were seen in human syphilitic sores a search for the parallel development of the homologue in guinea-pigs was made, and these bodies were found by J. W. Cropper in the large mononuclear cells of the peritoneal cavity of infected guinea-pigs; and he has now observed their complete development exhibiting stages exactly similar to those of the development of the pear-shaped bodies observed in human syphilides. This discovery in the guinea-pig's cells appears to give credit to the suggestion that the spirochaetes are really microgametes or male elements, and if this should prove to be the case, the possibility of some of the round or pear-shaped bodies being macrogamates or female elements must be considered. This, however, could only be proved by observing the act of conjugation, and convincing oneself of the observation. But further evidence exists as to this parasite being a protozoon having a cycle of sporogony in the fact that in the deeper layers of the secondary syphilides peculiar cells are to be found. These vary in size from that of a large blood platelet to that of an enormous cell larger than the eisinophile. These cells are spherical, are completely studded with multitudes of small chromatin copper-coloured staining granules. They have been likened to mast cells, but the variation of their size, their bag-like shape, the numbers of granules and absence of nuclei serve to distinguish them. I have only seen these cells in syphilitics.

7.—The History of the Parasite of Syphilis.

Klebs[1] appears to have been the first to consider the parasite of syphilis in 1879; Losdorfer[11] and Döhle[12] also described parasites. But Stassano[13] seems to have been the first to find cell inclusions in the manifestations of the disease. Then Siegel[14] described his *Cytoryctes luis*. In that description he pictures certain flagellate bodies, the counterpart of which can be seen by the jelly method. These flagellated bodies very closely resemble the production of the spirochaete-like forms, but appear outside the cells. Schaudinn went to investigate Siegel's work and he discredited it. A few months later he produced his famous discovery[15] of spirochaetes swimming free in syphilides, and these were named *Spirochaeta pallida*, and have been generally accepted as the causative agents of syphilis. Schaudinn's work was confirmed and proved by Metchnikoff and Roux.[16] It seems probable, however, that Stassano, Siegel, and Schaudinn can each claim to have discovered a phase of a parasite found in syphilitics, though I understand that Siegel has lately modified some of his former views. It is important to mention that de Korte,[17] while house-surgeon at the London Lock Hospital, described in 1906 free round bodies in the chancres of syphilitics, and "a cyst measuring 12μ, containing crystalline-looking threads, the whole staining very faintly." He called these protozoa, and classed them with the Ciliata; his illustrations are very poor. Recently I have heard that Mr. McDonagh, F.R.C.S., surgeon to the London Lock

E. H. ROSS: AN INTRACELLULAR PARASITE IN SYPHILIS DEVELOPING INTO SPIROCHAETES.



Hospital, has found cell inclusions in syphilis. Intracellular stages of various other spirochaetes have been described by many authorities—Leishman, Andrew Balfour, Fantham, Hindle, Blanc—but the relations between the various bodies described above and the *Spirochaeta pallida* were not hitherto demonstrated.

DESCRIPTION OF PLATE.

Fig. 1.—A small intracellular parasite commonly found in syphilitic chancres.

Fig. 2.—Division of the parasite chromatin; an inclusion found frequently in the mononuclear cells of the glands.

Fig. 3.—A cell from a chancre containing two parasites showing development. Note the copper colour of the parasites.

Fig. 4.—A mononuclear cell from a chancre containing three parasitic inclusions, two of which show the development of the spirochaetes, the third being an early stage of the phase shown in Fig. 6.

Fig. 5.—A large mononuclear cell found in the finger blood of a case of secondary (macular) syphilis. It shows the formation of the spirochaetes from a central chromatin mass within the included parasite.

Fig. 6.—A parasitic inclusion found in a cell of the blood taken from a syphilitic papule; it exhibits the subdivision of the chromatin of the parasite and the mode of the production of the free forms.

Figs. 7, 8, 9, 10.—The free forms—round and pear-shaped bodies—found commonly in the chancres, sores, and glands of syphilitics; they are amoeboid, stain in a characteristic manner, and are found in the earliest thickening which initiates the Hunterian chancre, as well as in all the manifestations of secondary syphilis examined up to the present time; they contain granules and a central chromatin mass, which at first sight appears as a vacuole containing a central dot, but if watched on the jelly it will be seen to stain as in Fig. 8.

Figs. 1, 2, 3, 6, 8 very closely resemble the development of the female element of *Lymphocytozoon cobayae*, the homologous parasite of guinea-pigs. The rabbit parasites show similar phases.

Figs. 3, 4, 5 very closely resemble the development of the spirochaetes or male element of *Lymphocytozoon cobayae* and of *Spirochaeta lumbrici*, the homologous parasites of guinea-pigs and earthworms. The rabbit parasites show similar phases.

Figs. 11 to 15.—Peculiar bodies found in secondary and early tertiary syphilis. They occur frequently in the sores, non-suppurating glands, and have been seen in the blood of two tertiary manifestations. Similar bodies to these are commonly seen in the blood of guinea-pigs infected with *Lymphocytozoon cobayae*, where they appear attached to mononuclear leucocytes, and in the seminal fluid of earthworms infected with *Spirochaeta lumbrici*, where they appear free as pictured here. In the guinea-pig these large granular bodies produce buds, each one of which, when separated, contains chromatin.

Fig. 16.—Two red blood corpuscles drawn for size comparison.

The figures are copied from specimens of living cells stained *in vitro* by the jelly method.

8.—The Technic of the Jelly Method.

The jelly method, as invented by H. C. Ross,[18] is as follows: 3 c.cm. of a 2 per cent. solution of agar in water, boiled and filtered; 1 c.cm. of Unna's polychrome methylene blue (Grübler), which has been previously diluted with two volumes of water—that is, 1 in 3; and 2 c.cm. of a solution containing 4·5 per cent. sodium citrate, 1·5 per cent. sodium chloride, and 0·225 per cent. atropine sulphate. This is boiled up together in a test tube and 0·3 c.cm. of a 5 per cent. solution of sodium bicarbonate ("alkali solution") added. Then a drop of this mixture when molten is poured on to a microscope slide and allowed to spread thereon, to cool, and to set. The chancre to be examined should be pricked with a large needle, and the blood squeezed from it so as to obtain the deeply situated cells. A drop of this blood—which, for convenience of conveyance and for the prevention of clotting, may be citrated with an equal quantity of a 3 per cent. solution of sodium citrate and a 1 per cent. solution of sodium chloride—is placed upon a cover-glass and inverted on to the set jelly. The blood spreads out between the cover-glass and the jelly in a thin film. In five minutes, when the cells have come to rest, the specimen can be examined under the microscope. The presence of the atropine in the jelly is optional; but it causes the cells to exhibit amoeboid movements, and this serves to differentiate the living cells from the dead ones, and the stain enables the cell contents to be distinguished clearly. The granules of the leucocytes stain scarlet, while the cells themselves are still motile; then as the nuclei stain—at first a pale blue, but later a deep ruby red—the motility ceases for the cells die. At length the stain fades from the cells, some of which may burst, for the cytoplasm liquefies and the cell becomes disorganised. It is not easy to make bad specimens by this method, and the pictures obtained are clear, each cell standing alone.

The parasites described generally stain after the granules of the leucocytes, and they appear as copper-coloured bodies either free—when they exhibit amoeboid movements—or included within the larger mononuclear cells. Each parasite shows the copper-coloured cytoplasm which contains a varying number of granules which stain deeply. In the centre of the parasite its "nucleus" appears, at first unstained, when it may be seen as a clear space showing a black dot in its interior. If the parasite is watched, however, the chromatin of the nucleus will be observed to stain suddenly a deep mauve, and then as the parasite dies the stain fades, leaving it a colourless sphere. This achromasia occurs to all the cells, and it is therefore essential to observe the specimen early; but the included parasite sometimes retains its stain after its host-cell has become achromatic. Occasionally in the external sores of syphilis the free parasites may be found engulfed by the polynuclear leucocytes. But in the non-suppurating glands and in the peripheral blood the larger mononuclear cells contain them, and it is in these cells that the development of the parasite may be observed; as stated before, however, the numbers of infected cells are much smaller in the peripheral blood than in the glands and sores.*

Some warnings are necessary. If the blood has been shed some time, or if there is too much alkali solution in the jelly, the cells may show diffusion vacuoles—small pink-staining dots, which may be mistaken for parasites; they possess no chromatin, however—indeed, they contain no structure, and they grow in size as the specimen stands until they burst. The parasite, on the other hand, has a clear cell wall, and possesses definite internal protozoal structure. If the patient from whom the blood is obtained has received treatment, such as an injection of "606," or of mercury, the free parasites are reduced in numbers, apparently within a few hours, whereas the included forms remain as before. This observation is based on but a few cases, and may require modification hereafter.

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* Similar parasites to those found in syphilis have been found in the blood of rabbits. These rabbits exhibit chancres, buboes, ulcers of the genitals, mouth, anus, etc., and some have white patches of the liver like the infected guinea-pigs The rabbit parasites, which are very numerous in the sores, possess phases indistinguishable from the human parasites.

9.—A Possible Means of Diagnosis.

It is too soon to be certain whether the presence of this parasite in the sores or blood can be regarded as a means of diagnosing syphilis. The evidence at present is strong. It has been found in syphilis only so far. The parasite develops into spirochaetes closely resembling the *Spirochaeta pallida.* The homologous parasite of guinea-pigs also develops similarly into spirochaetes. The homologous parasite of earthworms also develops similarly into spirochaetes. Guinea-pigs infected with *Lymphocytozoon cobayae* and the resulting spirochaetes exhibit gumma-like tumours, in which the parasites are found. The history of the finding of the parasites and its homologues points to its connection with the causative agent of the disease. Still, I cannot say yet with absolute certainty that this protozoon is the cause of syphilis. The quickest way of proving this point will be the examination of as many cases as possible, and if others will co-operate in doing this it will soon settle the matter. By this means we shall obtain the real value of the finding of this parasite as an aid to diagnosis. I have found them in the early induration which initiates the formation of the Hunterian chancre; so far, from that time onwards, they have been found in all primary and secondary sores examined.

10.—A Working Hypothesis.

Perhaps I may be permitted a suggestion. There seems justification for the suggestion that the guinea-pig or rabbit parasites might produce in man a mild affection which would modify the human disease of syphilis in the same way as the vaccination of cow-pox has modified small-pox in the individual and in the community. It must be remembered that the believed parasite of small-pox and vaccinia—the *Cytoryctes variolae* and *vaccinae* of Guarnieri—belongs to a family of cell inclusions (Chlamydozoa) somewhat similar to those I have described. Therefore the suggestion seems worth following up.

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N° 39

THE PRESENCE OF INTRACELLULAR AND FREE AMOEBOID PARASITES IN NOGUCHI'S CULTURES OF *SP. PALLIDA.*

In the British Medical Journal of December 14th last we described certain intracellular parasites found commonly in syphilitic lesions. These parasites were demonstrated by the jelly method of *in vitro* staining; and their development into spirochaetes was also described. They have now been found by us, and their presence has been confirmed by others, in every case of syphilis examined—in the primary and secondary lesions, in the circulating blood of syphilitics, and in some tertiary manifestations. In addition to these intracellular parasites, free amoeboid developmental forms were mentioned as occurring in chancres, glands, and sores. Analogous parasites found in certain animals suffering from allied affections were also noted (in rabbits, hares, guinea-pigs, earthworms). Lastly, these parasites were given the generic name of Lymphocytozoa, all species of which possess a spirochaete-like phase, the spirochaetes being the gametes.

As soon as Noguchi's cultures of the *Spirochaeta pallida* could be obtained a search was made in them for the parasites described. The medium employed by Noguchi consists of a mixture of agar and ascitic fluid in which are suspended small masses of rabbit tissue cells, and the spirochaetes grow anaërobically. After the sixth day of incubation, in the tissue cells (in those cultures which show the spirochaetes) what appear to be intracellular parasites have been found, and these seem to be indistinguishable from those seen by us in chancres, glands, sores, and blood of syphilitics. Moreover, the same free amoeboid forms, as described by us in the paper mentioned, have been found in the cultures. The cultures under observation were subcultures taken from those obtained from the Rockefeller Institute, New York.

The method of demonstrating these intracellular and extracellular parasites in the cultures is as follows: A subculture is chosen which contains spirochaetes. A long sterile pipette, with its upper end sealed, is thrust down through the layer of paraffin used to keep the medium anaërobic, through the semi-solid mixture of agar and ascitic fluid, into the mass of tissue cells at the bottom of the tube. The sealed end of the tube is then cut off, and

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some of the cells, some parasites, and perhaps some spirochaetes, enter the lower end. The upper end of the pipette is then sealed again, and it is withdrawn. Finally, some of its contents are shaken out on to a cover-slip, and this is inverted on to a ready-prepared jelly when the cells and parasites are stained by the jelly method. In old cultures, where there is considerable destruction of tissue, the parasites appear to undergo development outside the cells. The parasites in the cultures seem to be morphologically similar to those described in the BRITISH MEDICAL JOURNAL of December 14th, 1912, and pictured therein.

EDWARD HALFORD ROSS,
E. JENNINGS, Lieut.-Colonel, I.M.S.
(From the laboratories of the John Howard McFadden Researches.)

THE FATTY ACIDS OF BUTTER

By IDA SMEDLEY (*Beit Memorial Research Fellow*).

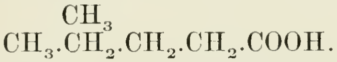
From the Bio-Chemical Department, Lister Institute

(*Received September 19th, 1912*)

I. The Nature of the Caproic Acid present in Butter

In spite of the large number of analyses of butter fat carried out for technical purposes, there remains considerable uncertainty as to the constitution of the fatty acids it contains. Even the nature of the lower fatty acids present is not definitely established, for whereas the caproic acid isolated from butter fat is described in most of the standard works of reference[1] as iso-caproic acid on the alleged authority of Chevreul, Lewkowitsch[2] prefers to regard it as the normal acid from analogy with the other fatty acids in butter. No work on the constitution of the hexoic acid present in fats appears to have been carried out since its isolation by Chevreul[3].

In Chevreul's pages no reference to the molecular structure of the acid is to be found, nor was the existence of two isomeric caproic acids established until fifty years after this work was published. The iso-acid was the first hexoic acid to be synthesised; Frankland and Kolbe[4] saponified the cyan amyl obtained from the amyl alcohol of fermentation and obtained an acid possessing, therefore, the structure



$$\mathrm{CH_3.\overset{CH_3}{CH_2}.CH_2.CH_2.COOH.}$$

It was not until twenty years later than the normal acid was discovered by Lieben and Rossi[5], who carefully characterised the normal and iso-acids[6], the solubility of the barium and calcium salts being especially characteristic. Up to this time, caproic acid had been found in butter[3], in cocoanut oil[7], in the flowers of *Satyrium hircinum*[8], in the fusel oil of beet-molasses[9], and in the fruits of *Ginko biloba*[10]. In none of these papers is any attempt made to identify this acid as either normal or iso, and, apparently, it is only because the iso-acid was synthesised twenty years before the normal that this error has crept into the literature. Franchimont and Zincke ascribe the normal structure to the hexoic acid obtained by oxidising the hexyl alcohol from Heracleum[11], and the caproic acid from the butryic fermentation of sugar was also identified as the normal acid by Lieben[12].

Examination of the data given by Chevreul indicates that the hexoic acid of butter has probably the normal structure; he gives as the solubilities of the barium and calcium salts:—

100 parts of water at 10·5 dissolve 8·02 parts Ba salt.
,, ,, ,, ,, ,, 14 ,, 2·04 ,, Ca ,,

Lieben and Rossi[6] give the following values:—

Normal Acid—

100 parts of solution at 18·5 contain 8·4967 grs. Ba salt.
,, ,, ,, ,, ,, ,, ,, 2·707 ,, Ca ,,

Iso-acid—

100 ,, ,, ,, ,, ,, ,, 34·65 ,, Ba ,,
,, ,, ,, ,, ,, ,, ,, 11·3 ,, Ca ,,

Chevreul's data appear, therefore, to indicate that the acid which he obtained from butter possessed the normal structure.

The identification of the acid present in natural fats as normal or as iso-caproic acid is one of considerable importance; the existence of iso-caproic acid $(CH_3)_2.CH.CH_2CH_2COH$ would suggest its derivation from leucin by deamidisation and, hence, might indicate a close connection with protein metabolism. This view was put forward by Felix Ehrlich[13], who suggested that the caproic acid present in neutral fats and that formed in fermentation, although hitherto regarded as the normal acid, might be mixtures of inactive di-methyl butyric with active methyl ethyl propionic acid, derived, respectively, from leucine and iso-leucine.

Ehrlich pointed out that fatty acids have been observed in the bacterial decomposition of proteins, leucine and caproic acid occurring together[14]; whilst the production of dextro-rotatory caproic acid from the decomposition of casein and elastin has also been observed by Neuberg[15]; in the former of these papers, however, no evidence is given as to the structure of the caproic acid present.

On the other hand, if the fatty acids which occur in butter and in cocoanut oil containing 4, 6, 8, 10, 12, 14, 16, 18 and 20 carbon atoms, respectively, consist of normal chains and are synthetic products, their formation from some simple starting product by an analagous series of reactions in which at each step two carbon atoms are added, appears probable.

It appeared desirable, therefore, to settle quite definitely the structure of the hexoic acid present in butter. The differentiation of the normal and iso series of acids is conveniently based on the examination of the amides, those formed from the iso-acids melting at considerably higher temperatures than those which belong to the normal series.

This is shown in the following table:—

Normal	M. Point	Iso	M. Point
Butyric	115°	Dimethyl acetic	128°
Valeric	114-116	Iso-propyl acetic	127
Caproic	100	Iso-butyl acetic	118
Heptoic	96-97		
Caprylic	97-98	Ethyl-butyl acetic	102
Nonoic	99		
Caprin	108		
Undecanic	103		

The isolation of the caproic acid was carried out as follows:—

About 3,500 grs. of pure butter fat* having the constants Iodine value 38·7, Saponification value 232, Reichert-Meissl 29·9, were saponified with alcoholic potash and the fatty acids liberated. The mixture was then steam-distilled until the odour of the lower fatty acids was no longer markedly perceptible in the distillate. The distillates, neutralised with potash, had a distinct odour of the higher fatty alcohols; sufficient was not, however, obtained for further identification. After concentrating to a small bulk, the solution of the soaps was acidified and extracted with ether. 125 grams of acid thus obtained were distilled from a flask fitted with a Young's 8-pear fractionating column and separated into the following fractions:—

Boiling point	Weight	
Up to 160°	24 grs.	
163—168	43 ,,	Boiling point, butyric acid, 163 (7 mm.)
168—173	25 ,,	
173—178	10 ,,	Boiling point iso-valeric acid, 174
178—183	3 ,,	
183—188	2 ,,	Boiling point N. valeric acid, 186
188—193	2 ,,	
193—198	0·5 ,,	
198—203	2 ,,	Boiling point iso-caproic acid, 200
203—208	5 ,,	Boiling point N. caproic acid, 205
208—213	6 ,,	

The residue remaining in the flask (16 grs.) was then distilled without a fractionating column.

213—218°	1·2 grs.	
218—228	4·8 ,,	
228—238	2·7 ,,	
238—248	1·5 ,,	
248—258	1·7 ,,	
Residue	4·1 ,,	Solidified on cooling

Two grams of each of the fractions boiling at 173-178°, 183-188°, 198-203°, 203-208° and 208-213° were then converted by Aschan's method[16] into the corresponding amides. From each of the fractions 198-203°, 203-208°

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* Pure butter was obtained from Lovegrove's Dairy, Checkendon.

and 208-213° an amide separated, melting at 96-97°. This was recrystallised from dilute alcohol and, finally, from a mixture of chloroform and petroleum; the crystals melted at 99-100°.

Amides were then prepared from Kahlbaum's normal synthetic caproic and isobutyl acetic acids. After recrystallisation these melted at 99-100° and at 118°, respectively. The melting point of the former was not depressed when mixed with the amide of the caproic acid from butter. The fraction boiling from 198-213° contained, therefore, normal caproic acid, and gave no indication of the presence of the iso-acid.

The fraction boiling up to 160° was now examined for acetic acid. It consisted chiefly of ether, and was neutralised by the addition of 1·2 c.c. N.KOH. On acidifying the solution of the small amount of potassium salt, drops of an insoluble acid with the characteristic butyric odour at once separated, so that the quantity of acetic acid, if any were present, could only have been exceedingly small.

In the above experiment, the steam-distillation was not continued until the whole of the volatile acids had passed over, but was stopped when 4·5 per cent. of the total weight of acid had been received. The proportion of acids present in the distillate examined was approximately:—

Butyric acid	85 gr.	74·6 per cent.
Caproic acid	13 ,,	11·3 ,,
Caprylic and Capric acids	12 ,,	10·5 ,,
Solid acids	4 ,,	3·5 ,,

II. THE EXISTENCE OF UNSATURATED ACIDS IN BUTTER OTHER THAN OLEIC ACID

A careful fractionation of the methyl esters of the butter acids was undertaken in order to obtain evidence as to the possible existence of lower members of the oleic acid series. It is a remarkable fact that whereas the 18-carbon atom unsaturated acid is commonly identified in fats, the existence of lower members of the oleic series has only been shown in cod-liver oil[17]. The experiments now carried out appear to indicate the presence of a small quantity of a lower unsaturated acid accompanying the decoic acid present.

The butter used for the preparation gave the following values: Reichert-Meissl 26·84, Iodine 37, saponification 228.

1,900 grams of dried butter-fat were shaken up with 567 c.c. of a solution of sodium in methyl alcohol (4·536 N). The emulsion formed was allowed to stand overnight and the esters next morning extracted with ether. After drying and evaporating off the ether on a water bath, 1,630 grams of liquid remained.

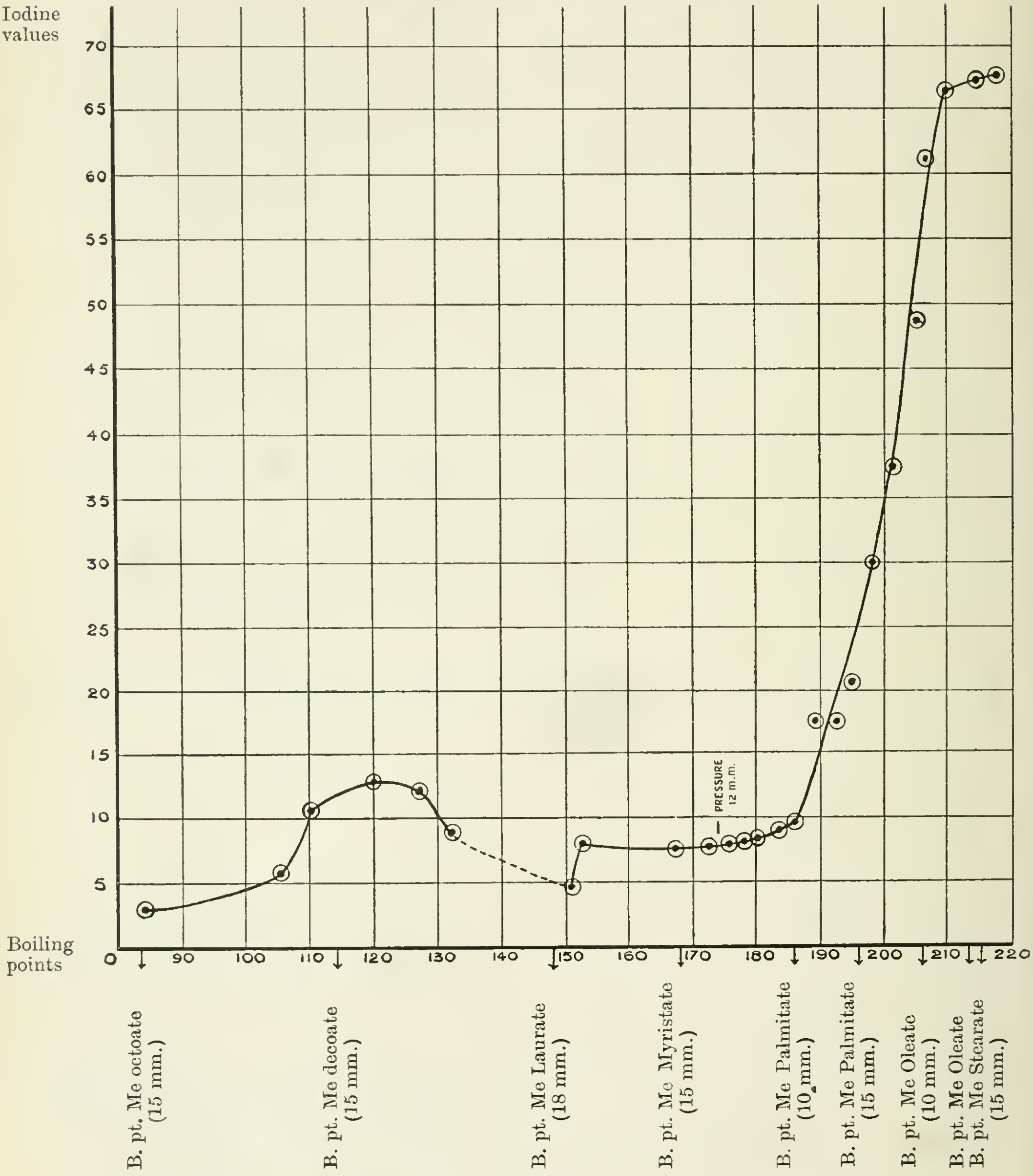
Examination for unsaturated acids.

The esters boiling above 160° under atmospheric pressure were then distilled under a pressure of from 10-15 mm., in a flask fitted with a Young's eight-pear fractionating column. The fractions were collected at first over from five to ten and then over every three degrees, and the iodine values of the different fractions determined; these are shown in the following table:—

Pressure	Boiling point	Weight of distillate	Iodine value	Saponification number	
15 mm. ...	80—85°	4 gms.	2·9	—	Boiling point methyl caprylate, 83° (15 mm.
	85—90	6 ,,	—	—	
	90—95	—	—	—	
	95—105	7 ,,	5·4	—	
	105—110	8 ,,	10·4	—	
	110—120	10 ,,	12·5	308·2	Boiling point methyl caprate, 114° (15 mm.) Sapon. No. 302
	120—127	10 ,,	12·0 11·9	307·3	
	127—132	8 ,,	8·7	282·9	
	132—147	—	—	—	
	147—151	10 ,,	4·9	—	Boiling point methyl laurate, 141° (15 mm.) Sapon. No. 262
	151—152 152—160	18 ,,	7·8	—	
	160—167	20 ,,	7·5	256·8	
	167—172	27 ,,	7·7	240·7	Boiling point methyl myristate, 167-8° (15 mm.) Sapon. No. 232
	172—175	16 ,,	7·9	—	
10 mm. ...	165—170	25 ,,	8·1	—	
	170—173	40 ,,	8·8	241	
	173—176	16 ,,	8·0	—	
12 mm. ...	176—180	5 ,,	8·1	—	
	180—183	14 ,,	8·8	—	
	183—186	23 ,,	9·4	—	
	186—189	100 .,	17·2	—	
	189—192	120 ,,	17·5 17·2	221	
	192—195	89 ,,	20·5	—	Boiling point methyl palmitate 186° (10 mm.) 196° (15 mm.) Sapon. No. 208
	195—198	74 ,,	29·9	215	
	198—201	29 ,,	37·2	—	
	201—204	68 ,,	48·5	—	
	204—207	138 ,,	61·1	196	
	207—210	215 ,,	66·6	—	
	210—215	114 ,,	67·3	—	Boiling point methyl oleate 205-6° (10 mm.) 212-3° (15 mm.) Boiling point methyl stearate 214-5° (15 mm. Sapon. No., methyl stearate, 188
	215—218	26 ,,	67·8	—	
Residue in flask boiling above 220		32 ,,	—	—	
		1272			

Distillation of Methyl Esters of Butter-Fat (obtained by Bull's method), under 10-15 mm. pressure.

Curve showing relation of Iodine values to Boiling points.



The curve shows a marked rise in the iodine values of the fractions boiling from 105-127°. The marked maximum at 120° is followed by a gradual depression until 150° is reached, the iodine value remains constant whilst the boiling-point rises through 30° C., and then a rapid increase marks the distillation of the oleic acid. So far, attempts (based on the crystallisation of the lead, magnesium and barium salts) to isolate unsaturated esters from these lower boiling fractions have proved unsuccessful. Were oleic the only unsaturated acid present, a steady rise in the iodine value, with increase of boiling-point, might have been expected. The increase in iodine value in the fraction boiling from 110° to 130° appears to the author to furnish evidence of the presence of an unsaturated ester containing a less number of carbon atoms than oleic, possibly a decylenic acid since it passes over with the fraction containing methyl decoate (caprate). It is possible that traces of other unsaturated acids may also be present, and that the iodine values found for the lower fractions may not be due to traces of oleic acid.

Distillation of the ethyl esters.

A confirmatory experiment was carried out in which the fatty acids from the 3,500 grams of butter-fat used for the separation of caproic acid (cp. p. 453) were converted into the ethyl esters by boiling for several hours with an alcoholic solution of hydrochloric acid. After washing with a dilute solution of sodium carbonate to remove free fatty acids, and drying with $CaCl_2$, 2,662 grams of esters were obtained, which were distilled under a pressure of 20-30 mm. and divided into three fractions:

(1) boiling up to 200° - - 281 grams;
(2) boiling from 200-220° - - 654 grams;
(3) boiling above 220° - - 1,727 grams.

The fraction boiling up to 200° was then distilled under a pressure of from 20-28 mm. in this case a six-pear fractionating column being used. The ethyl esters boil about 20° higher than the corresponding methyl esters. The iodine and saponification values of the different fractions were determined as in the previous experiment; a distinct increase in the iodine value was found in the fraction, the saponification value of which agreed with that required for the decoic ester.

Distillation of ethyl esters of butter fatty acids boiling up to 200° (20-30 mm.); (the more volatile acids having been partially separated).

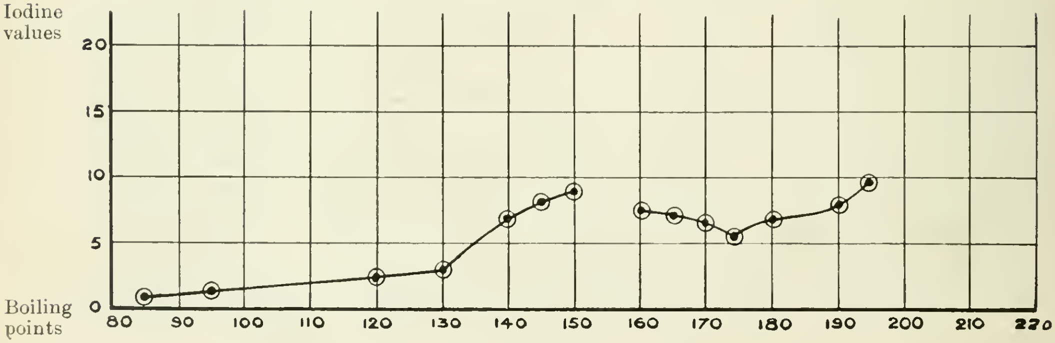
Pressure	Boiling point	Weight of Fraction	Saponification Number	Iodine Number	
25-28 mm. ...	80—85°	12·8	389·8	0·7	Sapon. No. ethyl hexoate = 388 (caproate)
	85—90	2·3	—	—	
	90—95	2·0	—	1·5	
	95—100	0·2	—	—	
	100—105	1·2	—	—	
	105—110 110—115	0·2	—	—	
	115—120	4·0	349·6	2·5	Sapon. No. ethyl octoate = 326 (caprylate)
	120—125 125—130	20·0	316·5	3·0	
	130—135	5	—	—	
	135—140	5·5	—	6·8	
	140—145	15·5	288·8	7·8	
	145—150	21	282·1	8·7	Sapon. No. ethyl decoate = 280 (caprate)
* 20 mm. ...	150—155	—	—	—	
	155—160	18	259·4	7·2	
	160—165	9	—	6·8	
	165—170	7	252·8	—	
	170—175	6	—	5·5	Sapon. No. ethyl laurate = 245
	175—180	18	238·9	6·5	
	180—185	3	—	—	
	185—190	8	—	7·4	
	190—195	14	222·6	9·6	Sapon. No. ethyl myristate = 219

The distillation was not further continued.

* Distillation stopped here: continued on the following morning under a pressure of 20 mm.

Distillation of Ethyl Esters of Butter-Fat (obtained by esterification of fatty acid with ethyl alcohol and HCl) under 20-25 mm. pressure.

Curve showing relation of Iodine values to Boiling points.



Three other experiments were carried out, in each of which between 300 and 400 grams of methyl esters were prepared and distilled. In every case the iodine values of the fractions corresponding to the decoic acid were higher than those of the succeeding fractions. The increase is not very large, but was present in five different specimens of butter examined, and it may be inferred therefore that it is generally present, and may be regarded as significant. It appears probable that the presence of a decylenic acid is indicated.

III. On the Presence of Stearic Acid in Butter

In view of the statements of Lewkowitsch and others (cp. Lewkowitsch, loc. cit.) that, when examined by the ordinary methods used in the examination of butter, only traces of stearic can be found, the higher boiling fractions of the methyl esters were examined for stearic acid. From the fractions in two experiments boiling at 210-215° a solid ester was separated, which, when twice re-crystallised from alcohol, melted at 37-38°, and on saponification gave an acid crystallised from alcohol in plates melting at 69°, and which was therefore identified as stearic acid. The quantity of stearic present was approximately estimated as about 10-15 per cent. of the total butter acids. Lewkowitsch has drawn attention to anomalies observed in the estimation of stearic acid when lower acids than palmitic are present (cp. Lewkowitsch, loc. cit., Vol. I, p. 453). The presence of considerable quantities of stearic acid in butter-fat was also found by Caldwell and Hurtley[18], who state that the oleic acid of butter occurs chiefly in combination as an oleo stearo palmitin.

Reaction with sodium-nitro-prusside.

The distillate, boiling up to 140° under atmospheric pressure, gave a very low iodine value of between two and three units. The fraction boiling from 100-120° was heated with a solution of caustic potash, acidified with acetic acid and a solution of sodium-nitro-prusside and strong ammonia then added; a deep violet colour developed. The same fraction gave negative results when treated with Fehling's solution and with ammoniacal silver nitrate. The sodium-nitro-prusside reaction was obtained in two out of five experiments in which the esters had been prepared by Bull's method; but when the esters were prepared by an acid method of alcoholysis, in no case was the reaction obtained. This reaction is especially characteristic of aceto-acetic acid and of acetone, and it is possible that these were present in those samples examined which gave positive results. In view of the inconstancy of the results obtained, it is possible that these products had been formed as the result of bacterial action. Further evidence as to the nature and origin of the substances producing this reaction is desirable, as the certain detection of aceto-acetic acid in butter would be a point of considerable importance.

Conclusions

1. No evidence of the presence of acetic acid was found in the butter examined.

2. The hexoic (caproic) acid present in butter possesses the *normal* structure; no indication of the presence of the iso-hexoic acid was obtained. It seems probable that of all the naturally existing specimens of caproic acid which have been described, only that occurring in the bacterial decomposition of proteins has a branched structure.

3. The proportion of stearic acid was estimated as from 10-15 per cent.

4. Evidence was obtained of the existence of lower members of the oleic acid series; the iodine value of the decoic ester fraction is appreciably greater than those of the fractions immediately preceding or following it. This may be regarded as indicating the presence of a lower unsaturated acid, possibly of a decylenic acid.

5. The sodium-nitro-prusside reaction, characteristic of aceto-acetic acid and of acetone, was given by the butyric ester fraction obtained from two out of five samples of butter, esterified by sodium methylate at ordinary temperature: the remaining three samples gave negative results. The reaction may possibly have owed its origin to the products of bacterial action; it was observed in two cases where Haller's acid method of esterification had been used.

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Journal Physiology, Vol. XLV.

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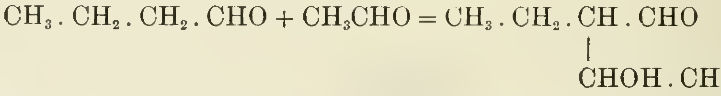
The biochemical synthesis of fatty acids from carbohydrate. By IDA SMEDLEY.

Two alternative suggestions have been put forward as to the method by which fatty acids may be formed in the animal organism from carbohydrate: (1) that these acids are formed by condensation of sugar molecules with subsequent reduction and oxidation; thus by condensation of two pentose and one hexose molecules, the precursor of palmitic acid would be formed, whilst from three hexose molecules stearic acid would be derived; (2) that the fatty acids are built up from simple decomposition products of the carbohydrates, condensation of some 2-carbon atom compound occurring in successive stages. The presence in butter of fatty acids containing all even numbers of carbon atoms from 4 to 20 supports the second of these hypotheses, if the assumption be allowed that the butter acids are the results of a synthetic process. Nencki suggested acetaldehyde as the decomposition product of sugar which might form straight chain fatty acids by a series of aldol condensations

$$2CH_3CHO = CH_3 . CHOH . CH_2 . CHO \longrightarrow CH_3 . CH_2 . CH_2 . COOH.$$

Against this view it has been urged that when the higher fatty aldehydes are condensed in vitro with acetaldehyde, branched chains of carbon atoms are formed; thus



$$CH_3 . CH_2 . CH_2 . CHO + CH_3CHO = CH_3 . CH_2 . \underset{\displaystyle CHOH . CH_3.}{\underset{|}{CH}} . CHO$$

Raper showed that aldol is capable of condensing with itself in alkaline solution to form a normal 8-carbon atom chain from which by subsequent oxidation and reduction normal caprylic acid may be obtained. I showed subsequently that crotonaldehyde behaves similarly:

$$2CH_3 . CHOH . CH_2 . CHO$$
$$= CH_3 . CHOH . CH_2 . CHOH . CH_2 . CHOH . CH_2 . CHO.$$

As however neither the condensation of aldol nor of crotonaldehyde appeared to fulfil the conditions necessary to furnish an analogy with the formation of fatty acids in the organism, I have now studied other methods of synthesising fatty acids which might afford more hopeful analogies for biochemical syntheses. A satisfactory method has been found in the condensation of aldehydes with pyruvic acid which takes place at ordinary temperature in dilute alkaline solution:

$$RCHO + CH_3CO . COOH = RCHOH . CH_2 . CO . COOH.$$

The α keto acids formed are oxidised by Ag_2O in alkaline solution thus:

$$RCHOH . CH_2 . CO . COOH + O = RCHOH . CH_2 . COOH + CO_2.$$

Benzaldehyde, butyl, crotonyl and isovaleryl aldehydes have been found to react in accordance with the above equations.

Neuberg and Hildesheimer have shown that pyruvic acid is fermented by yeast with production of acetaldehyde and CO_2. It is possible that a similar enzyme exists within the liver-cell which splits up the α keto acid into aldehyde and CO_2, the aldehyde at once condensing with a molecule of pyruvic acid to form a higher keto acid, which may either be oxidised by an oxidase to the fatty acid containing one carbon atom less (a process accomplished in vitro by the action of Ag_2O) or may be again transformed by enzymes into an aldehyde capable of reacting with another molecule of pyruvic acid.

$$CH_3CO\,.\,COOH = CH_3\,CHO + CO_2.$$
$$CH_3CHO + CH_3CO\,.\,COOH = CH_3CHOH\,.\,CH_2\,.\,CO\,.\,COOH.$$
$$CH_3CHOH\,.\,CH_2\,.\,CO\,.\,COOH + O = CH_3CHOH\,.\,CH_2COOH + CO_2.$$

The displacement of the β hydroxyl group by a hydrogen atom would result in the formation of a saturated fatty acid containing two more carbon atoms than the aldehyde from which it was formed.

In support of the hypothesis now put forward the following points may be urged:

(1) There is some evidence that pyruvic acid occurs in the animal organism as a decomposition product of carbohydrate. Fellner has shown that if a liver rich in glycogen be perfused with blood containing ammonia, alanin may be isolated from the product and pyruvic acid is suggested as the intermediate substance since Embden and Schmitz have shown that ammonium pyruvate, perfused through the liver is converted into alanin.

(2) Knoop has shown that if γ phenyl α amino butyric acid be fed to a dog, a considerable proportion of the acid appears in the urine as its acetyl derivative; the same phenomenon was observed by Neubauer and Warburg in their perfusion experiments. There is some reason to believe that the acetylating agent may be pyruvic acid (cf. Knoop *Ztschr. f. physiol. Chem.* LXVII. (1910) 489).

(3) The production of fat from carbohydrate and from protein in the organism is explained if the substance condensing to form the fatty acid chains (pyruvic acid) occurs as a decomposition product both of carbohydrate and of protein. The formation of alanin from pyruvic acid in the animal organism has been established by the work of Knoop, Neubauer and Embden and his co-workers, whilst Neubauer has also shown the probable production of α keto acids from the amino acids of protein in the formation of fusel-oil by yeast.

Neither pyruvic nor any other α keto acid has as yet been detected in the tissues; their existence within the living cell as intermediate products of metabolism seems however possible.

NcH

[*Reprinted from the* PROCEEDINGS OF THE ROYAL SOCIETY, B. Vol. 86]

The Chemical Action of Bacillus cloacæ (*Jordan*) *on Citric and Malic Acids in the Presence and Absence of Oxygen.*

By JAMES THOMPSON.

(Communicated by Arthur Harden, F.R.S. Received August 27,—Read November 14, 1912.)

(From the Biochemical Department, Lister Institute.)

The chemical action of *Bacillus cloacæ* on citric acid and on malic acid has not up to the present been investigated.

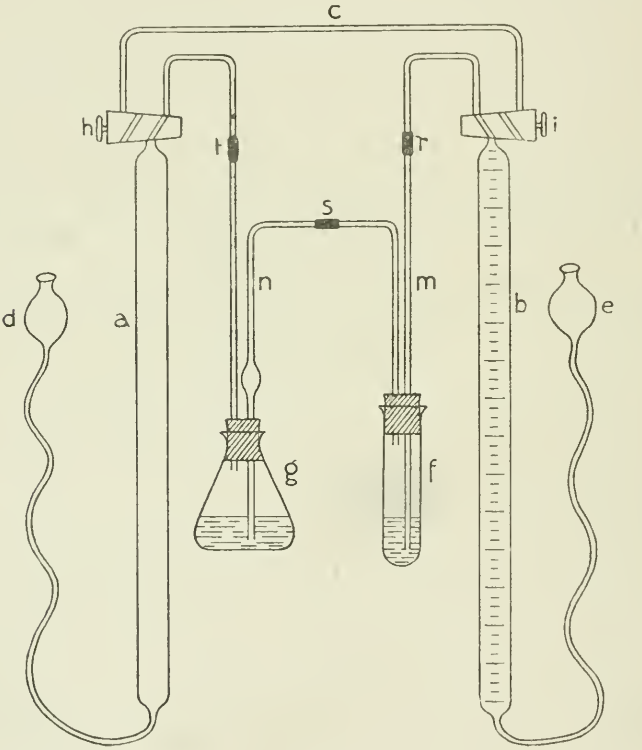
Since this organism is a facultative anaërobe, the experiments were undertaken with the object of ascertaining the effect of the presence of oxygen on the course of the fermentation produced. It was found, however, that the organism could not grow on ammonium malate in the absence of oxygen, so that the observations on this subject were limited to the case of citric acid.

Emmerling (1) has described the decomposition of malic acid by *B. lactis aërogenes* (Escherich), a very closely allied organism, and Bosworth and Prucha (2) have shown that, during the souring of milk, the citric acid present in fresh milk is converted by *B. lactis aërogenes* into acetic acid and carbon dioxide.

I. In investigating the chemical changes produced by the action of *B. cloacæ* on various media, the determination of the respiratory coefficient was first undertaken. For this purpose the apparatus described below, and shown diagrammatically in the annexed figure, was used.

Two burette tubes (*a*) and (*b*), fitted with two-way taps (*h*) and (*i*), are connected by a capillary tube (*c*). To each are attached by stout rubber tubing the pear-shaped receivers (*d*) and (*e*). The incubation tube (*f*), containing the medium under examination, and the flask (*g*), containing 50 c.c. of a 10 per cent. solution of caustic potash, are connected with the taps of the burettes as shown in the diagram, by stout rubber connections at (*t*) and (*s*). The internal capacity of the flask and incubation tube and connecting tubes between the taps (*h*) and (*i*) was determined. Deducting the volume of medium and caustic potash solution, the volume of air contained in them at the beginning of the experiment is known.

The burettes and receivers having been charged with mercury, the incubation tube (*f*), containing 10 c.c. of the medium, previously sterilised and subsequently inoculated from a culture of *B. cloacæ* on the same medium, is connected to the burette (*b*) and the absorption flask (*g*). The tap (*i*) is removed, and, about 80 c.c. of air having been allowed to flow into the graduated burette, the tap is replaced in connection with the tube (*f*). By suitable manipulation of the receivers (*d*) and (*e*) and the two-way taps, the liquid in the tubes (*m*) and (*n*) is brought to the level of the liquid in (*f*) and (*g*). The volume of air, thus brought to atmospheric pressure, is then read off on the burette (*b*), the temperature and barometric pressure being carefully noted. The tap (*h*) is now opened to the flask (*g*), the



receiver (*d*) being slightly lowered to create a partial vacuum in the burette tube (*a*). At intervals of 24 hours, the air in the burette (*b*) is swept through the apparatus into the burette (*a*) through the tube (*m*) by raising the receiver (*e*) and simultaneously lowering the receiver (*d*). Then, by reversing the taps, it is returned to the burette (*b*) through the tube (*c*).

By thrice repeating these manipulations, the carbon dioxide produced by the growth of the organism in the medium is removed from the tube (*f*), and absorbed by the caustic potash solution in (*g*). At the conclusion of the experiment, indicated by the non-diminution of the volume of air in (*b*), the flask (*g*) is disconnected, and the carbon dioxide determined by double titration with N/10 H_2SO_4, using phenolphthalein and methyl orange as indicators. The experiments were carried out at an approximate temperature of 37°.

It is essential that the burette taps be perfectly gas-tight. The form shown in the diagram was found to be the most satisfactory and the taps used were tested against a vacuum. As a lute, resin ointment proved to be the best for a temperature of 37°.

In making the readings, of which the initial and final are, of course, all important, it is essential to be quite sure that no marked alteration of temperature has taken place previous to observation. To obviate any error from this source, three readings were made in each case at intervals of 30 minutes, and their agreement showed that the experiment was being satisfactorily carried out.

To test the apparatus, a blank experiment was made, 10 c.c. of water being placed in the tube (*f*).

Internal capacity of apparatus = 176·8 c.c.

	Burette reading.	Temperature.	Barometer reading.	Volume of air at N.T.P.
	c.c.	°	mm.	c.c.
June 17, 12.30 P.M.	83·2	37·6	756·6	213·0
2.30 ,, ...	83·0	37·4	757·0	213·2
5.0 ,, ...	83·0	37·4	757·1	213·2
,, 19, 10.30 A.M.	82·8	37·8	758·7	213·0
The door of the hot room was allowed to remain open for 5 minutes. Fall in temperature 4·2°, rising quickly on again closing the door.				
June 19, 12.0 noon	80·2	35·4	758·7	214·3
3.0 P.M.	83·0	37·6	757·6	213·1

From this test it is seen that the apparatus described is quite satisfactory provided that due precaution is taken that the burette readings are made while the apparatus is not subject to fluctuations of temperature.

Throughout the experiments detailed below, the air was circulated through the apparatus every 24 hours, readings being taken to check the course of the experiment. At the conclusion of each experiment, the residual gas in the apparatus was examined gasometrically for marsh gas and hydrogen, which were in each case found to be absent.

II. For the examination of the products resulting from the action of *B. cloacæ* on citric and malic acids, the general method described below was employed. The media were prepared from the acids by drying these to constant weight *in vacuo* over sulphuric acid, dissolving in distilled water, and, after neutralising with ammonia to convert them into their ammonium salts, adding sufficient normal sulphuric acid to give the finished product an acidity of +15. Acid potassium phosphate was added in the proportion of 0·1 grm. per litre of medium. The malic acid was present in the proportion of 6·7 grm. per litre, while, for the citric acid medium, the proportion used was 7 grm. of crystallised acid per litre, this quantity corresponding to 6·4 grm. of the anhydrous acid.

One litre of the medium was placed in a 2-litre flask, fitted with leading and delivery tubes plugged with cotton-wool. The delivery tube was connected outside the incubator with an absorption flask provided with a soda-lime tube, to allow of exit of the excess of air or nitrogen, and containing 200 c.c. of a 10 per cent. solution of caustic potash for the absorption of the carbon dioxide produced. After sterilisation, the medium was inoculated from a pure culture of *B. cloacæ* on agar, and a slow current of air, free from carbon dioxide, was bubbled through the apparatus during the course of growth. In the case of the experiments conducted in the absence of oxygen, the leading tube of the incubation flask was fitted with a three-way tap, by means of which a stream of nitrogen could be passed through the flask, after first displacing the air in the leading tube. The nitrogen was prepared by the action of ammonia solution on copper turnings, and passed through wash-bottles containing sulphuric acid, chromous chloride solution, and, finally, caustic potash. Growth in the incubation flask having ceased, which was found to be the case after about four weeks, the amount of carbon dioxide was determined by double titration of the caustic potash solution, and the contents of the incubation flask were examined as follows: The liquid was made up to 1 litre with distilled water, 500 c.c. were taken, made acid with 40 c.c. of normal sulphuric acid, and carefully distilled. The first 200 c.c. of distillate were collected, titrated with normal potash, using phenolphthalein paper as indicator, and redistilled. One hundred cubic centimetres of distillate were collected, and its specific gravity determined. In each experiment this was found to be equal to that of water, proving the non-production of any notable quantity of alcohol. The distillation of the original liquid was continued with steam, until titration of the distillate showed no further neutralisation of alkali. The combined titrated distillates were evaporated to low bulk, made up to 100 c.c. with distilled water, and examined for volatile acids. Formic acid, when present, was determined by heating 10 c.c. of the solution for two hours on the water-bath with 10 c.c of a 10 per cent. solution of sodium nitrite and 50 c.c. of a saturated solution of mercuric chloride, and weighing the precipitated mercurous chloride. From the weight found, the amount of formic acid was calculated.

The identity of the acids was established by determination of the molecular weight by means of the barium salt. The residue in the distilling flask was concentrated to 100 c.c., and completely extracted with ether in a continuous extraction apparatus. The ether was distilled off, and the residue dried at 100° and weighed. The identity of this residue with succinic acid was established by converting it into its barium salt. A weighed portion was heated for one hour on the water-bath with water and excess of barium carbonate. After filtration, the insoluble portion was well washed with hot water and the filtrate made up to 200 c.c.; 100 c.c. of this solution were evaporated to dryness, dried, and weighed. Conversion of this residual barium salt into barium sulphate showed it to consist of barium succinate, and proved that lactic acid is not produced by the growth of *B. cloacæ* on either malic or citric acid. Further proof that the residue was succinic acid was afforded by the determination of its melting point and by its reaction with ferric chloride.

The amount of residual acid, *i.e.* the acid not used up by the organism, was determined in the residue from which the succinic acid had been extracted with ether. This was made faintly alkaline with caustic potash, and evaporated to low bulk to get rid of ammonia. The residue was then taken up with water, exactly neutralised with acetic acid, and barium acetate added. Four volumes of 95 per cent. alcohol were then added, and the liquid allowed to stand overnight. In the case of the malic acid only a faint precipitation took place, showing that all but traces of the malic acid had been used up. In the case of citric acid, the precipitated barium salt was filtered off, washed with 63 per cent. alcohol, ignited, and finally weighed as barium sulphate, from which the amount of citric acid left was calculated.

In no case did the fermentation liquids give Vosges and Proskauer's reaction, thus proving that methylacetylcarbinol, which is produced by the action of *B. cloacæ* on mannitol and glucose, is not formed.

III. *Determination of the Respiratory Coefficient.*

The following tables show the results obtained with the apparatus previously described. In each case 10 c.c. of medium were employed. For Experiment 1 the figures are given in full, in order to show the course of the experiment; in the following experiments only the initial and final readings are given, though it is to be noted that they were similarly conducted. The day-by-day readings are essential, as the observation of any marked irregularity in the consumption of oxygen would point to some leak in the apparatus. The experiments were continued until the readings after an interval of 24 hours were the same.

Experiment 1.—Peptone Water.

	Internal capacity of apparatus.	Burette reading.	Temperature.	Barometer reading.	Volume of gas, corrected for N.T.P.
	c.c.	c.c.	°C.	mm.	c.c.
May 7 ……	176·8	87·0	38·2	761·6	216·7
„ 8 ……	„	87·2	37·8	759·1	216·7
„ 9 ……	„	85·8	38·0	758·3	215·0
„ 11 ……	„	80·2	36·8	762·4	213·4
„ 12 ……	„	76·0	34·6	757·3	211·5
„ 13 ……	„	76·4	36·2	756·1	209·3
„ 14 ……	„	74·3	35·2	753·0	208·0
„ 15 ……	„	71·2	35·6	755·7	205·7
„ 18 ……	„	64·8	37·0	774·3	203·7
„ 19 ……	„	65·8	37·4	771·0	203·0

Oxygen absorbed………… $216·7 - 203·0 = 13·7$ c.c.

CO_2 produced …………… 10·35 c.c.

Respiratory coefficient … $\dfrac{10·35}{13·7} = 0·76.$

No. of experiment.	Substance.	Corrected volume of gas.		Oxygen absorbed.	CO_2 produced.	Respiratory coefficient, CO_2/O_2.
		Initial.	Final.			
		c.c.	c.c.	c.c.	c.c.	
2	Peptone …	214·1	207·3	6·8	6·5	0·96
3	Malic acid	219·6	209·1	10·5	17·9	1·7
4	„	221·7	211·3	10·4	17·9	1·7
5	„	216·1	204·0	12·1	20·2	1·67
6	„	221·2	210·2	11·0	18·4	1·67
7	„	216·3	204·9	11·4	15·7	1·4
8	„	214·0	209·9	4·1	6·7	1·64
9	Citric acid…	220·3	214·6	5·7	13·4	2·35
10	„ …	206·4	196·6	9·8	31·3	3·2

The mean result of the experiments gives for malic acid a respiratory coefficient of 1·63. This is in good agreement with the value found from a reaction proceeding as follows :—

$$3C_4H_6O_5+5O_2 = 2C_2H_4O_2+8CO_2+5H_2O,$$

which gives a respiratory coefficient of 8/5 = 1·6.

Experiments 9 and 10 were carried out with citric acid. The results are discussed later on (p. 9).

IV. *Chemical Action of* Bacillus cloacæ *on Malic Acid in the Presence of Oxygen.*

1. The only products of the action of *B. cloacæ* on malic acid in the presence of oxygen were found to be carbon dioxide, acetic acid, succinic acid, a small amount of a fatty substance, the nature of which was not determined, and traces of alcohol. The following tables give the results obtained. Table I gives the actual weight, on the malic acid used up, of the various substances produced, while Table II gives the number of carbon atoms per molecule of malic acid represented by each product :—

Table I.—Percentages.

	a.		*b.*	
	grms.	per cent.	grms.	per cent.
Carbon dioxide	3·40	50·8	3·25	48·5
Acetic acid	1·06	15·8	1·09	16·3
Succinic acid	2·02	30·2	2·36	35·2
Total	6·48	96·8	6·70	100·0

Malic acid consumed = 6·70 grms.

Table II.—Carbon Atoms.

	a.	*b.*
Carbon dioxide	1·54	1·48
Acetic acid	0·71	0·73
Succinic acid	1·37	1·79
Total	3·62	4·00

Malic acid = $C_4H_6O_5$.

In three further experiments, the carbon dioxide and acetic acid only were determined, the following results being obtained:—

	c.	*d.*	*e.*	
Carbon dioxide	2·86	2·36	2·39	grms.
Acetic acid	1·21	0·70	0·82	„

The molecular ratios $CO_2/C_2H_4O_2$ calculated by the formula $\frac{CO_2}{44} \times \frac{60}{C_2H_4O_2}$ are:—

Experiment.........	*a.*	*b.*	*c.*	*d.*	*e.*
Molecular ratio, $\frac{CO_2}{C_2H_4O_2}$	4·25	4·06	3·21	4·62	3·99

The action of *B. cloacæ* on malic acid in the presence of oxygen probably goes on in two ways: the one, an oxidation of the acid to carbon dioxide and acetic acid by atmospheric oxygen, and the other, an oxidation accompanied by reduction of a portion of the malic acid to succinic acid. The degree of access of the oxygen during fermentation will thus account for the difference in the proportions of succinic acid formed.

Assuming that growth in a sufficient supply of oxygen leads to complete oxidation of the malic acid to carbon dioxide and acetic acid, the following equation may be put forward as representing the changes which take place:—

$$3C_4H_6O_5 + 5O_2 = 2C_2H_4O_2 + 8CO_2 + 5H_2O.$$

This gives a ratio $CO_2/C_2H_4O_2$ of 4 and a respiratory coefficient of $8/5 = 1·6$, the latter being in good agreement with the results of the experiments previously described. In these experiments it is to be noted that a small quantity (10 c.c.) of medium is in contact throughout with a good supply of air, so that oxidation may be considered to be almost complete. If any oxidation were due to the oxygen derived from a second molecule of malic acid, the respiratory coefficient would be increased. On the other hand, oxidation of the acetic acid would decrease the coefficient. The numbers actually found were 1·67, 1·67, 1·7, 1·7, 1·64 and 1·4.

V. Contrary to the statement of Ritter (2), it was found that *B. cloacæ* would not grow on ammonium malate in the absence of oxygen.

VI. *Chemical Action of* B. cloacæ *on Citric Acid in the Presence of Oxygen.*

The substances produced from citric acid by *B. cloacæ* in the presence of oxygen were found to be the same as from malic acid. The amount of acetic acid produced is markedly greater in the former case, and the molecular ratio $CO_2/C_2H_4O_2$ for malic acid is four times as great as that for citric acid. Table III gives the results of an experiment showing the actual weights and percentages of the substances found, and the corresponding number of carbon atoms. In Table IV are given the results of three further experiments, in which the succinic acid and residual citric acid were not determined.

Table III.

	Grammes.	Percentages.	Carbon atoms.
Carbon dioxide	2·40	37·8	1·63
Acetic acid	3·36	53·0	3·39
Formic acid.........................	0·07	1·2	0·05
Succinic acid	0·19	3·0	0·19
Residual citric acid..............	0·06		
Total..........................	6·08	95·0	5·26
Citric acid used up = 6·4—0·05 = 6·35 grm.			

Table IV.

	b.	*c.*	*d.*
	grm.	grm.	grm.
Carbon dioxide	3·48	2·58	2·73
Acetic acid	3·04	3·52	3·16
Formic acid	None	None	None

From these results the molecular ratios were calculated.

Experiment..................	*a.*	*b.*	*c.*	*d.*
Molecular ratio, $\frac{CO_2}{C_2H_4O_2}$	0·97	1·56	1·0	1·18

VII. *Action of* B. cloacæ *on Citric Acid in the Absence of Oxygen.*

In contrast with the growth of *B. cloacæ* in the presence of oxygen, that in absence of the latter results in the production of a small amount of formic acid. There is also an increased production of acetic acid, and the molecular ratio $CO_2/C_2H_4O_2$ diminishes markedly. Tables V and VI give the results of two experiments; in a third experiment (*c*), the carbon dioxide and acetic cid only were determined.

Table V.—Percentages.

	a.		*b.*	
	grm.	per cent.	grm.	per cent.
Carbon dioxide	1·64	28·8	1·84	31·2
Acetic acid	3·50	61·3	3·44	58·5
Formic acid	0·15	2·6	0·22	3·7
Succinic acid	0·17	3·0	0·03	0·5
Total	5·46	95·7	5·53	93·9
Citric acid used up	5·70	—	5·88	—

Table VI.—Carbon atoms.

	a.	*b.*
Carbon dioxide	1·26	1·36
Acetic acid	3·92	3·74
Formic acid	0·11	0·15
Succinic acid	0·19	0·03
Total	5·48	5·28
Citric acid = $C_6H_8O_7$.		

In a third experiment the carbon dioxide and acetic acid only were determined.

c.	Carbon dioxide	1·76 grms.
	Acetic acid	3·62 „

From these three experiments the following molecular ratios are obtained:—

Experiment	*a.*	*b.*	*c.*
Molecular ratio $\frac{CO_2}{C_2H_4O_2}$	0·64	0·73	0·66

Neglecting the small amounts of formic and succinic acids, this ratio corresponds with the following equation for the anaërobic decomposition of citric acid by *B. cloacæ*, in which the molecular ratio

$$CO_2/C_2H_4O_2 = \tfrac{6}{9} = 0{\cdot}67.$$

$$(1)\quad 4C_6H_8O_7 + 4H_2O = 9C_2H_4O_2 + 6CO_2.$$

The effect of the presence of oxygen is to increase the relative proportion of carbon dioxide with respect to the acetic acid. This may be due to the occurrence of reactions such as the following:—

$$(2) \quad 4C_6H_8O_7+2H_2O+2O_2 = 8C_2H_4O_2+8CO_2+2H_2O.$$

$$(3) \quad 4C_6H_8O_7+4O_2 = 7C_2H_4O_2+10CO_2+2H_2O.$$

These give the ratios shown in the following table, which also includes the numbers actually found:—

	Molecular ratio, $\frac{CO_2}{C_2H_4O_2}$.	Respiratory coefficient, $\frac{CO_2}{O_2}$.
Equation 1	0·67	
2	1·0	4·0
3	1·4	2·5
Found by experiment	0·97	
	1·0	
	1·56	
	1·18	
	—	2·35
	—	3·2

It is also possible that the decomposition takes place in the same way in the presence or absence of oxygen, according to equation (1), but that in presence of oxygen a portion of the acetic acid is subsequently oxidised.

Summary.

(*a*) Malic acid is not ermented by *B. cloacæ* in the absence of oxygen.

(*b*) Malic acid is decomposed by *B. cloacæ* in the presence of oxygen into carbon dioxide, acetic acid, and succinic acid, with traces of alcohol. The decomposition probably goes on in two ways; oxidation by atmospheric oxygen to carbon dioxide and acetic acid, and oxidation at the expense of another portion of the malic acid, which is thereby reduced to succinic acid.

With a good supply of air the respiratory coefficient CO_2/O_2 and the molecular ratio $CO_2/C_2H_4O_2$ found agree well with the values given by a reaction proceeding as follows:—

$$3C_4H_6O_5+5O_2 = 2C_2H_4O_2+8CO_2+5H_2O.$$

(*c*) In contradistinction to malic acid, citric acid is fermented by *B. cloacæ* in the absence of free oxygen. In addition to carbon dioxide, acetic, and succinic acids, the products resulting from aërobic fermentation, formic acid is produced, while there is an increased production of acetic acid.

The molecular ratio $CO_2/C_2H_4O_2$ found agrees with the value for the following equation:—

$$(1) \quad 4C_6H_8O_7+4H_2O = 9C_2H_4O_2+6CO_2.$$

(*d*) Citric acid is decomposed by *B. cloacæ* in the presence of oxygen into the same products as malic acid.

The values found for the respiratory coefficient and for the molecular ratio $CO_2/C_2H_4O_2$ are intermediate between those required for the following equations :—

$$(2)\quad 4C_6H_8O_7 + 4O_2 = 7C_2H_4O_2 + 10CO_2 + 2H_2O.$$

$$(3)\quad 4C_6H_8O_7 + 2H_2O + 2O_2 = 8C_2H_4O_2 + 8CO_2 + 2H_2O.$$

This is probably due to the difficulty of maintaining complete aëration of the medium during the experiment, the decomposition being therefore partly anaërobic and partly aërobic in character.

(*e*) It is possible that the decomposition of citric acid by *B. cloacæ* takes place in the same way in the presence or absence of oxygen, according to equation (1), but that in the presence of oxygen a variable portion of the acetic acid is subsequently oxidised.

(*f*) Methylacetylcarbinol, which is produced by the action of *B. cloacæ* on mannitol and glucose, is not formed in the fermentation of malic acid or of citric acid by this organism.

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On the Morphology of the Leishmania of Italian Kala-Azar.

Third Communication[1]: Cytological Researches on Leishmania in Cultures.

By

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With Plates 19 and 20.

RESEARCHES on the minute structure of Protozoa, although initiated in comparatively recent times, have already contributed to science a considerable number of facts of great interest, especially from the point of view of general cytology. But while observations conducted with the most delicate methods of cytological technique are feasible up to a certain point when dealing with Protozoa of a certain size, they meet with no slight difficulties when dealing with micro-organisms which, like the species of Leishmania, are of the smallest dimensions. When, more than a year ago, I made successful cultures of Leishmania on the medium of Novy, McNeal and Nicolle, by means of splenic juice obtained by puncture of the spleen of a young kala-azar patient of Bovalino Calabro, my teacher, Prof. Grassi, repeatedly recommended me to undertake the present investigations with the special object of studying the little-known structure of the blepharoplast and the rhizoplast, with regard to which Grassi, in collaboration with Foa, has described a whole complicated system of organellæ in Jœnia, Trichonympha, and other Protozoan parasites of Termites.

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[1] The author's two previous communications on this subject were published in the 'Archiv f. Schiffs- und Tropenhygiene,' xiv (1910), Beiheft 4, and 'Pathologica,' iii (1912).

My investigations, begun in the Laboratory of Comparative Anatomy at Rome, were continued by me during my stay in the Institut für Schiffs- und Tropenhygiene at Hamburg, and were resumed, after some interval, in the Protozoological Department of the Lister Institute of London by the express desire and advice of Prof. Minchin. To Profs. Grassi and Minchin, who have directed and guided me in a field of study almost new to me, I desire to express my gratitude.

Investigations on the finer structure of Leishmania in the Leptomonas form are almost entirely new, especially for the Leishmania which is the specific agent of kala-azar in the countries of the Mediterranean basin and which goes under the name of L. infantum. None of those who have occupied themselves with the morphology of this Protozoon in the flagellate stage have made use of those methods of histological technique which alone permit a study of the minute structure of Protozoa, and have limited themselves rather to describing the various forms which are met with in the culture-tubes, some of them trying especially to reconstruct the developmental cycle of the parasite. But the data with regard to the developmental cycle of the parasite are more than ever uncertain and contradictory; as is known for other flagellate Protozoa, the cultural forms are for the most part aberrant or resting forms, and it is probable that many of those described in the cultures of Leishmania may be regarded rather as degenerative forms. On the other hand, concerning the structure of the parasite there have resulted many inexact observations.

Of great interest are, above all, the questions regarding the significance of the blepharoplast and its exact relations with the flagellum. Rogers has described in the first days of the development of L. donovani in acidulated citrated blood, a body which stains with eosin, for the most part rounded or oval, closely apposed to the blepharoplast, which is now attached to the periphery or approximated to it. This "eosin-body" (named by Leishmann "flagellar body") is found in the elongated forms at the anterior extremity of the parasite, always maintaining the same relations with the blepharoplast. It could be better described as an area coloured in pink around the blepharoplast and the rhizoplast, and it is supposed by Franchini to have been found ultimately expelled from the parasite in the dried smears fixed in alcohol and stained with Giemsa's stain.

Wenyon also has occupied himself with the structure of L. tropica in cultures, and my researches, more complete in many points, will permit me, as will be seen in the sequel, to follow out still further the intimate morphological relations which exist between these two species, so closely allied, the one the specific agent of oriental sore, the other of kala-azar.

I have had at my disposition three sources of cultures. Besides that obtained by myself and mentioned above, I have received other cultures from the Pasteur Institute of Paris, which originated from Nicolle in Tunis, and others very recently from the Clinica Pediatrica of Palermo. To Messrs. Mesnil and Jemma, who so kindly sent me them, I return my best thanks.

The results obtained from these different cultures agree perfectly, as was to have been expected, so that in the descriptions of my observations I refer to the one or the other indifferently.

When preparations are to be made of flagellates in a culture on blood-agar, it is a most important precept that the smears should be very thin, otherwise the flagellates for the most part do not become differentiated, and Protozoa also stain in an incomplete manner; moreover the examination is hindered by the presence of hæmoglobin particularly, which takes an intense colour, and in which the Protozoa are imbedded.

The smears on a cover-glass or slide were made with a small drop of the culture by means of another cover-glass or slide, smearing the material in a backward direction.

The cultures were always very rich in parasites and of very recent date (from two or three days to a week), in order to avoid the presence of dead or altered forms and to obtain the greatest possible number of dividing forms.

It is an essential precept to fix the preparation immediately, before the smear dries, because, as in the case of very many other Protozoa, the Leishmanias become deformed rapidly, assuming always a form more stumpy or even rounded; the method of drying, besides itself altering the form and the structure, is not a sufficiently rapid means of fixation. It is for this reason that those authors who have made use only of dried smears, stained with Giemsa's stain, have given incomplete and inexact descriptions, as recently Franchini. In particular, with this method the rhizoplast is not always successfully demonstrated, and never the exact connection of the latter with the kinetonucleus.

For fixation I have made use largely of Schaudinn's alcoholic sublimate and of Maier's fluid, which is a slight modification of the former; of osmic acid, followed by subsequent fixation in methyl alcohol, absolute alcohol, or Schaudinn's fluid; and of Flemming's fluid and Hermann's fluid, always used cold. After fixation in mixtures containing sublimate, I have followed usually the technique indicated by Giemsa for wet smears and sections, that is to say, treatment with iodine solution and afterwards with hyposulphite; but I abandoned this procedure when, like Rosenbusch, using a solution of sublimate in which there were no crystals, I made the fixation last but a short time.

Two methods of staining have given me especially good results—Giemsa's method for wet smears and Heidenhain's classic iron-hæmatoxylin; I have not had equally good differentiation of the stain with the abbreviated procedure of Rosenbusch, which, however, gives good results and has the advantage of saving much time. The differentiation of the preparations is especially delicate and difficult, requiring to be checked by frequent examination under the microscope; for this purpose I have made use with advantage of the water-immersion objective of Zeiss (2·5 mm.), with which it is possible to control step by step the most minute structural details.

The cultures of Leishmania develop rapidly, and flourish in the Novy-McNeal-Nicolle medium prepared according to the formula of Nicolle, which, I think, it worth while to record briefly here:

Agar-agar, not washed	14 grm.
Sodium chloride	6 ,,
Distilled water	900 c.c.

The mixture is dissolved and sterilised in the autoclave, taking care to add afterwards some sterile water, as much as has been lost during the sterilisation. The agar, not alkalinised nor neutralised, is placed in the sterile tubes in the desired quantity (I make use of test-tubes 12 cm. in length and about 2 cm. in diameter, and place in each 4 c.c. of agar), and then sterilised afresh for ten minutes. The tubes can be kept a long time, provided that all desiccation of the agar is avoided by closing them with a rubber cap and keeping them in the ice-chest, sheltered from light.

At the moment of preparing the substratum the agar is redissolved by boiling water and allowed to cool to 45°–55° C., at which temperature is added to it, with proper precautions to ensure sterility, one third of its volume of defibrinated rabbit's blood, introducing it with a sterile pipette holding 10 c.c., which I change two or three times during the operation. The agar and the blood are mixed by shaking the test-tube well, but care should be taken that excessive froth is not formed. The tubes are then placed on an inclined plane in order that the blood-agar may solidify in a form like the mouthpiece of a flute, and left thus about twelve hours. Closed again with rubber caps they are placed in the incubator at 37° C., in order that the water of condensation may collect in good quantity. There is no advantage in leaving them in the thermostat two or three days, as Nicolle prescribes, since the water of condensation always diminishes a little.

Tubes prepared in this manner can be kept in the cold chamber at a temperature of about 0° C. for some months ready for use, but care must be taken to maintain them for half-an-hour at 21^{c} prior to planting them. Nicolle, Massaglia and others have employed aseptic puncture of the heart in order to obtain the rabbit's blood in a sterile manner. Although this method, after some experience, succeeds fairly well, it never allows so large a quantity of blood to be obtained as from the carotid, and I have adopted with constant success this procedure, which, moreover, is used also in Mesnil's service at the Pasteur Institute in Paris. The anæthetised rabbit is fixed in a manner which exposes the throat; the skin, previously disinfected and carefully shaved, is cut with aseptic precautions along the median line and the trachea is laid bare, taking care not to wound the blood-vessels; at the side of the trachea the carotid is easily found and is isolated from the nerves without injuring them, and is tied with sterile silk as near as possible to the skull. It is seen that at the level of the thyroid gland the thyroid artery arises from the carotid, and this is isolated and tied near the carotid and cut above the knot in order to have it as a firm stalk for holding and raising the carotid with a forceps later on, without diminishing its lumen by taking hold of its wall directly. As a greater precaution at this point I pour a little alcohol on the artery, and then I cut it near the cranial ligature and collect the jet of blood in a sterile Erlenmayer flask containing the usual glass beads in order to defibrinate the blood. With the forceps which holds the carotid by the stalk formed by the stump of the thyroid artery, the carotid can be held within the orifice of the Erlenmayer flask without touching its walls (which, moreover, are previously sterilised in the flame) until the blood is drawn.

By this method as much as 55–60 c.c. of blood can be obtained from a rabbit of medium size without the animal dying, if care be taken to tie the cut carotid quickly and to sew up the wound rapidly without chloroforming the animal too much. The operation, when a certain expertness has been acquired, may last only a few minutes.

It is necessary that I should make some remarks with regard to nomenclature, since the various structural elements of Leishmania have received various denominations, especially from the Italian authors; moreover, it is necessary to put aside names which have different meanings and may produce confusion, and to restore the nomenclature of Leishmania to that which is in scientific use for the cell-body of flagellate Protozoa allied to the genus Leishmania.

Since my observations, like those of Wenyon for Leishmania tropica, enable me to affirm the nuclear nature of the blepharoplast-nucleus of the Leishmania of Italian and Tunisian kala-azar in the flagellate form of the Leptomonas in cultures, one should abandon, as has been done for the other Protozoa, the names "nucleolus," "micronucleus," "centrosome" and "extra-nuclear centrosome," which record a past uncertainty of knowledge with regard to the blepharoplastic nucleus and engender a grave confusion with other constituent parts of the cell endowed with quite other functions. There remain in common use the denominations kinetonucleus and blepharoplastic nucleus or blepharoplast simply; but Minchin and Woodcock and other English authors indicate very properly by the name blepharoplast the basal granule (Rosenbusch) from which the flagellum takes origin, and which is situated usually in the zone of the nuclear sap of the kinetonucleus

The principal nucleus or trophonucleus (Woodcock) of Leishmania is of vesicular type, and, especially in the preparations fixed wet and stained with hæmatoxylin by Heidenhain's method (figs. 19–46), presents the typical structure of the nucleus of trypanosomes and other allied Protozoa. It is constituted by a membrane, well defined and of a certain thickness, which encloses a clear space, the so-called zone of nuclear sap, and at the centre a granule of chromatin stained a deep black and of compact structure, the karyosome. In the preparations differentiated for a long time in iron-alum, it is possible to distinguish in some Leishmanias a very small granule, the centriole, in the interior of the karyosome.

From the karyosome very delicate filaments (linin) can sometimes be seen to arise, connecting the karyosome to the nuclear membrane in a radiate manner, and showing here and there very minute granules connected to one another in their turn by transverse threads. On the inner surface of the nuclear membrane, granules and dots (Chromatinkomplex of Schaudinn) are observed united to the karyosome by delicate filaments. But in general the linin-framework and the chromatin of the nuclear sap-zone are very feebly developed in the principal nucleus of Leishmania and the granules extremely minute.

With the method of Giemsa, after wet fixation (fig. 3) it is also possible to demonstrate the minute structure of the nucleus in all its constituent parts; on the other hand, in dried smears Giemsa's stain usually colours the sap-zone also, so that the nucleus appears as a rounded homogeneous body, in the interior of which some granules of chromatin more intensely stained can be made out with difficulty.

In the smears stained with iron-hæmatoxylin there are sometimes found in the nuclear sap-zone of the principal nucleus one or even two granules intensely stained, larger than those which are met with normally along the threads of the linin-network and easily distinguished from them. Their number, one or two, is especially characteristic, and in fig. 42 I have represented a nucleus in which the two granules are connected by a very delicate filament, not to be confounded, it appears to me, with a filament of linin. We are dealing here, perhaps, with the centriole or extra-karyosomic centrosome dividing into two for the first time as a prelude to the division of the whole nucleus; or is this a phenomenon of chromatinic reduction, like others of which I shall give an account in the sequel?

The kinetonucleus, situated between the trophonucleus and the anterior extremity of the body, but nearer to the former than to the latter, appears composed mainly of a fusiform or oval body situated with its greater diameter in a transverse direction, compact and intensely stained, representing the karyosome of the kinetonucleus. This body presents itself sometimes as two rounded granules quite distinct and united by a zone stained more feebly, instead of as a single dense oval body; but in that case there exist already two rhizoplasts or even two flagella, and I believe that this is always a case of the beginning of the process of division, which commences, as is known, in the kinetonucleus.

Around the karyosome a clear circular zone is observed, corresponding to the nuclear sap-zone of the trophonucleus and delimited by a membrane, very delicate but always quite evident. In this zone it is sometimes possible to distinguish also some fine chromatinic granules without linin-threads. At the periphery of the clear zone there is found anteriorly as a rule a granule more or less evident, the basal granule or blepharoplast properly so-called, from which the rhizoplast arises.

The flagellum of Leishmania never ends at the anterior pole of the parasite, as has been described by observers whose methods of staining have been defective, but is always continued into the body of the Protozoon in a posterior direction and along the axis of the parasite as far as the kinetonucleus. There is thus a rhizoplast constantly present in the Leishmania, a structure which, moreover, exists already in the forms of Leishmania in the human body, as I have described and figured in one of my preceding publications.

To establish exactly the precise relations and connection of the flagellum with the kinetonucleus is not always easy.

In the greater number of cases the flagellum ends in the blepharoplast proper or basal granule, which, as I have stated, is found anteriorly in the sap-zone of the kinetonucleus, more or less near to the karyosome, sometimes adhering to it. When there is a certain amount of space between the two it is possible to demonstrate a structure in the form of a finely striated cone, as if it were constituted by very minute fibrils, which unite the karyosome to the blepharoplast itself. This is just as is observed sometimes in many trypanosomes and in L. tropica.

In other cases the flagellum is united directly to the kinetonucleus, and in many cases (figs. 5, 10–13, 26, 31) the impression is obtained that it arises directly from it. Then there is no basal granule in its usual position. The possibility cannot, of course, be excluded absolutely that in these cases the basal granule and point of origin of the flagellum are found in a slightly lower plane, behind the karyosome which lies over it, but the figures are very suggestive of the interpretation that the flagellum is really in continuity with the karyosome, within which in such cases the blepharoplast would also be found. Moreover, to the latter is attributed usually the significance of the centriole of the motor nucleus; and the assumption that the blepharoplast is found in some cases in the interior of the karyosome explains also, as we shall see in the sequel, the behaviour of these various elements in the process of division.

It is known that in Leishmania the process of division begins usually by that of the kinetonucleus; nevertheless there are cases in which a precocious multiplication of the principal nucleus takes place, so that there may be in the same parasite two trophonuclei completely distinct and the first stages of division of the motor nucleus. In the kinetonucleus the process begins by the division of the blepharoplast, followed immediately by that of the flagellum; in this way are produced forms in which, as in figs. 7, 8, 16, 18, there are two rhizoplasts which unite anteriorly, and which are continued by a single flagellum; in these cases either there are already two basal granules in the sap-zone, or the doubled rhizoplast is continued into the interior of the karyosome, and this is almost the rule.

The splitting of the flagellum proceeds rapidly, and from it results almost always a normal flagellum, and, near it and parallel to it, one much shorter (figs. 6, 9, 10, 11, 13, 14, 31, 33, 34, 35), as is observed frequently also in trypanosomes. In most cases, however, after the new rhizoplast has been formed, it detaches itself at its distal extremity from the other, and, when separated, gives origin to the new flagellum, which begins to protrude from the anterior pole of the parasite and subsequently grows continually in length (figs. 4, 5, 9, 10). At the same time or immediately afterwards the division of the karyosome takes place.

It should be noted at this point that I have never found true and proper figures of karyokinesis of the kinetonucleus with the formation of an achromatinic spindle.

All these stages of division are, of course, only to be observed in preparations fixed by wet methods and suitably stained; dried preparations give only a very inexact and incomplete idea of the whole process (figs. 6 and 7).

The successive stages of division of the kinetonucleus can be followed step by step in figs. 9–18 and 31–35. The karyosome becomes elongated and constricted in the middle, and the two halves which are thus produced travel further and further apart, remaining connected by a very slender filament, which persists during the division of the trophonucleus; in this way a true centrodesmosis of the kinetonucleus is formed. At this stage there exist already two rhizoplasts and two flagella, which usually become detached from the two halves, still connected, of the karyosome.

Special interest attaches to the Leishmania represented in fig. 34; in this specimen the two flagella take origin from two granules situated outside the two halves of the karyosome and respectively very close to each of them; a very delicate achromatinic filament unites the two granules or blepharoplasts properly so-called, from which, as I have said, the flagella originate. This would prove that the blepharoplasts have the significance of centrioles of the motor nucleus, and that actually in an early period of the division they are found in the interior of the karyosome, at the point when the flagellum of the flagella already formed seem to take origin from the interior of the karyosome itself; subsequently, however, the blepharoplasts regain their normal position in the sap-zone near the membrane.

The division of the principal nucleus follows a process perfectly analogous, and takes place in all its stages within the nuclear membrane. I believe that the appearances shown in figs. 28, 29 and 30 should be interpreted as early forms of multiplication. In these the nuclear chromatin is not collected, as in the majority of cases, in a compact mass forming a single karyosome at the centre, but is broken up into granules and short rods which perhaps correspond to chromosomes, and which, distributed at first irregularly in the sap-zone, afterwards arrange themselves so as to constitute a single elongated mass of irregular contour, disposed transversely to the longer axis of the organism and always contained within the nuclear membrane. Subsequently the chromatin collects at the extremities in two masses, which remain united by a thread that becomes continually more attenuated. Here also there is a true and proper centrodesmose with an achromatinic filament which stains strongly and resists prolonged differentiation with iron-alum; it unites, as is seen specially in fig. 35, two stained granules, which correspond, I believe, to the centrioles of the daughter-nuclei.

The nuclear membrane persists throughout and can be seen well in the preparations stained with iron-hæmatoxylin, and better still in those stained by Giemsa's method. In the latter it is seen that the membrane, like the whole nucleus, assumes at first an oval form, then is gradually constricted in half in the form of a figure of eight, disposed transversely to the greater axis of the Leishmania; when the two halves are joined by the delicate filament, it constitutes the membranes of the two rounded daughter-nuclei (figs. 11, 12, 13, 14, 15, 16, 17, 18, 32, 33, 34, 35). Between the latter the centro-desmotic filament breaks and the division is complete. Meanwhile the division of the protoplasm has already begun (fig. 17), or follows rapidly.

In spite of many long and patient observations I have never come across forms of division, either of the trophonucleus or the kinetonucleus, in which the presence was observed of an achromatinic spindle and of a typical equatorial plate, such as has been described in trypanosomes by Rosenbusch and some other authors, and I believe that I can exclude completely this type of the process of division in the case of Leishmania in the flagellated Leptomonas form. In this case there is a process of mitosis without formation of an achromatinic spindle giving rise to two daughter-cells exactly equal. I wished, however, to reproduce in the interesting figures 44, 45 and 46 some nuclei which present exactly the phenomena described recently by Horta and Machado (**15**) in Trypanosoma chagasi n. sp., a trypanosome of fishes in Brazil, and interpreted, I believe, erroneously as a complete process of heteropolar division. In support of my judgment there is also the fact that the authors cited have never seen two distinct nuclei arise from the supposed division, or subsequent fission of the protoplasmic body of the parasite; just as I have never found this to occur in the Leishmania.

I believe rather that we are dealing simply with a reduction of nuclear chromatin, with no direct relation to the process of multiplication. Without wishing to suggest phenomena of maturation in relation with a hypothetical sexual differentiation, I am persuaded that these facts of nuclear reduction (since it is a question without doubt of a reduction of chromatin) are to be placed rather in the group of phenomena which Richard Hertwig terms "Kernplasma-Relation" (**14**).

Having studied the process of division in many preparations, it has never happened to me to come across forms which would suggest that the kinetonucleus performs the function of a centrosome in the division of the principal nucleus, a function ascribed to it by Moore and Breinl, on which ground they have named the kinetonucleus the "extra-nuclear centrosome." The nuclear structure and the mode of division of the motor nucleus are sufficient to exclude such an interpretation, but it should also be recognised, from the examination of the preparations, that the division of the trophonucleus and that of the kinetonucleus appear up to a certain point to be independent the one of the other, so much so that the one may be already finished when the other is beginning, and if they take place synchronously, they may be found in different stages. Moreover, I have never observed a fusion of the kinetonucleus with the principal nucleus, as Row has described in the cultures of Leishmania tropica, not even in the first days of the development, when the flagellated forms are arising from those of the spleen.

With regard to the first origin of the flagellum my observations are very scanty, since I have only twice obtained cultures of spleen-material, but I am able to confirm the descriptions given by other authors, and to state precisely the manner in which the flagellum exists.

Beginning with the small forms of Leishmania from the internal organs of kala-azar patients, and especially those in which the rhizoplast can already be demonstrated (figs. 7, A, B, of my memoir [**26**]), the next stage has a more rounded form, or is already pear-shaped (see fig. 38) with a more distinct rhizoplast. The flagellum begins to protrude from the body of the parasite and becomes continually longer, while the organism becomes pyriform and elongated, and the kinetonucleus, which at first was found by the side of the nucleus, passes anteriorly to it. In this way there are flagellates of more or less elongated form with one end pointed and the other, the anterior end, rounded, and also long and very slender forms such as that represented in fig. 21.

The protoplasm of Leishmania in cultures appears, with the best fixatives, very finely granular, and contains frequently here and there granules of various sizes, identical with the volutin-grains of trypanosomes. After prolonged coloration with Heidenhain's hæmatoxylin of smears fixed in Schaudinn's fluid, the protoplasm often assumes a variegated appearance, with patches intensely coloured and separated by clear spaces.

I have never observed in the protoplasm the existence of a filament uniting the kinetonucleus to the trophonucleus, as described by Prowazek and some of his pupils, amongst them also recently Chagas in Trypanosoma cruzi. After fixation, especially with liquids containing osmic acid (osmic acid and Schaudinn's fluid, Flemming's and Hermann's fluids), I have observed the presence in the space between the trophonucleus and kinetonucleus and very close to the latter of a corpuscle with contours not very sharply defined, for the most part rounded and without an internal structure, and stained more feebly than the nuclear karyosome (see figs. 36, 37, 21). In preparations fixed with osmic acid and Schaudinn's fluid I have demonstrated fairly frequently a filament which, starting from the posterior pole of the parasite, traverses the body in a spiral and ends near the motor nucleus, in some cases in the body just mentioned, which in my opinion can be identified with that described by Novy (19) and by Sangiorgi (24) in trypanosomes.

I have been led to these investigations above all by the very important works of Grassi and of Foa on the structure of the Protozoa parasitic in Termites, studies which have opened an horizon of research with regard to the motor apparatus of flagellate Protozoa. I do not know whether the various parts described in the Leishmanias as rhizoplast and kinetonucleus can be considered analogous to the organellæ described by Grassi and Foa, and named by Janicki the "parabasal apparatus." It may be that the ill-defined body situated near the kinetonucleus can be compared to the parabasal body. But after the examination of many preparations with more or less effective methods of staining, I am certainly inclined at present to consider the spiral filaments demonstrated by me after osmic fixation rather as folds of the ectoplasm, artificial products of the preparation, than as true and proper structural elements.

I have not omitted to undertake repeatedly examinations in the fresh state in physiological salt solution, and also in the same liquid containing a very weak solution of picric acid, so dilute as to permit the flagellates to continue living for a certain time. The Leishmanias then show exactly the same form as in the stained preparations when the fixation has not been preceded by desiccation ; there is perhaps a still greater uniformity in the morphology of the parasite, with prevalence of the typical spindle form with the anterior extremity rounded, while the stumpy and rounded forms are rarer, those that occur being for the most part in process of division. In the interior of the body the trophonucleus and the kinetonucleus can be distinguished clearly, especially in forms which do not contain many granules.

It is a noteworthy fact that the two nuclei of the Leishmania present an identical appearance, as a dark point surrounded by a clear halo. When the motility of the Protozoa is diminished and the examination of the structural details is rendered possible, it is often possible to make out that the flagellum is continued as a rhizoplast into the body of the organism as far as the kinetonucleus, ending at the boundary of the clear halo of the latter. In the space between the trophonucleus and the kinetonucleus it is not infrequently possible to observe a body slightly darker than the protoplasm with contours not well defined, without a halo and without perceptible structure, exactly similar to the body which is made evident by the iron-hæmatoxylin stain, especially after osmic fixations. My attention, as is natural, has been drawn particularly to the search for something which would correspond to the axostyle ("mestolo," Axenstab), that is to say, the so-called axial rod in the protoplasm between the nucleus and the posterior extremity, but I have never succeeded in observing it. Perhaps the rhizoplast is homologous with it?

Observation in the fresh state and with very powerful magnification is of course very tiring, and it is above all very difficult to pick out details of structure in organisms so minute, but it is to be hoped that further researches will lead to new and definitive knowledge with regard to the finer structure of these flagellate Protozoa.

THE LISTER INSTITUTE,
LONDON;
May 20th, 1912.

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EXPLANATION OF PLATES 19 AND 20,

Illustrating Dr. Arrigo Visentini's paper "On the Morphology of the Leishmania of Italian Kala-azar."

PLATE 19.

Figs. 1–18.—Flagellated forms of Leishmania infantum from cultures stained with Giemsa's stain.

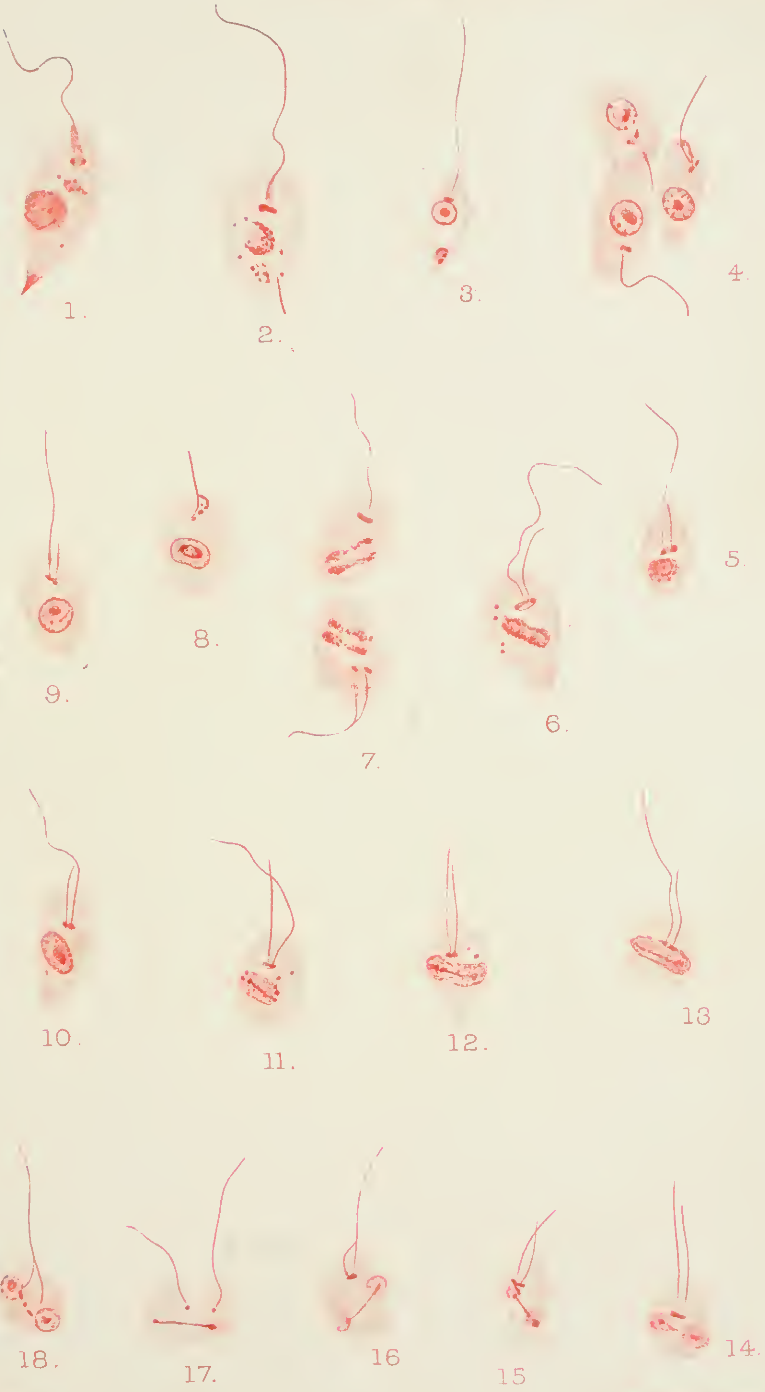
Figs. 4–18 show various stages in the process of division.

All figures drawn with the camera lucida at a magnification of 2000 diameters.

PLATE 20.

Figs. 19–38.—Flagellated forms from cultures stained with iron-hæmatoxylin, magnified 2000.

Figs. 39–46.—Nuclei of the flagellated forms drawn at a magnification of 3000 diameters.



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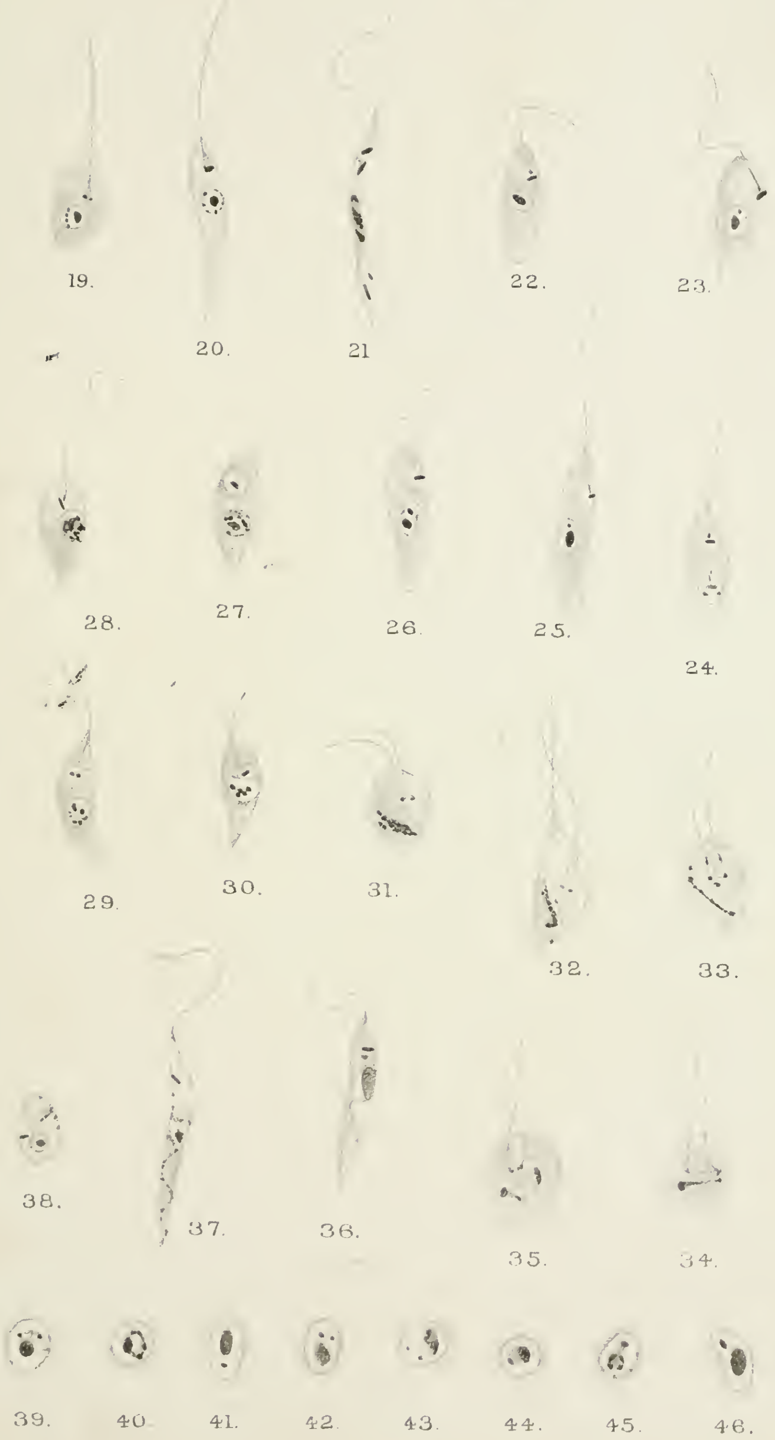
LEISHMANIA INFANTUM



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LEISHMANIA INFANTUM.

The Transmission of Leishmaniosis by means of Cultures, and the Mechanism of the Natural Immunity in Rats and Guinea-pigs.

By

Dr. Arrigo Visentini.

With Plate 21.

Attempts to transmit to animals the infection of Leishmania by means of cultural forms have not always had constant results up to the present.

Nicolle (1909) first inoculated dogs and monkeys (animals notoriously susceptible to leishmaniosis) with 1 c.c. of a culture, but without result; afterwards, however, in collaboration with Manceaux, he obtained positive results by inoculating Macacus cynomolgus and M. sinicus, reproducing the infection with the clinical picture of infantile leishmaniosis.

Novy (1908), by means of cultures obtained from Nicolle, had already succeeded in infecting dogs and afterwards other laboratory animals (?) by inoculating considerable quantities of the parasites, in the case of one dog about 270 cultures in fifty injections. Subsequently, however, Novy (1909) established the fact that a single injection of a suspension obtained from a score of culture-tubes is sufficient to produce leishmaniosis in dogs.

The rabbit has so far been proved to be susceptible only to corneal injection and to the forms occurring in the hæmatopoietic organs. Volpino, by scarification of the cornea with material obtained from a dog infected with experimental leishmaniosis, produced a keratitis similar, up to a certain point, to that of experimental syphilis. In the circumscribed corneal lesions provoked by Volpino there were found typical Leishmanias, contained in the large mononuclears.

Basile and I have repeatedly inoculated large quantities of cultures in various ways, including the corneal method, without succeeding in our object. On the other hand, a single intra-peritoneal injection of a culture of Leishmania at the eighth transplantation, about fifteen days old and very rich in flagellate forms, was found by Franchini[1] (1911) to be sufficient to produce in a guinea-pig an infection with Leishmania which ran a course that might be termed acute. After six days the animal began to lose flesh and to show febrile symptoms; after twenty-six days the depression previously evident had become very marked. The guinea-pig was sacrificed before spontaneous death intervened. Its weight had diminished by almost half of the original amount. At the autopsy the liver and spleen were found to be enlarged; the marrow of the long bones was very abundant and of a reddish-brown colour; the supra-renal capsules were also enlarged. In the spleen, liver, bone-marrow, peripheral blood, supra-renal capsules and kidney Franchini found forms of Leishmania more or less numerous, but always extracellular, a state of things which, in the author's opinion, is in relation with the septicæmic type of the infection.

In the guinea-pig, rat and mouse, Laveran and Pettit (1909) have obtained slight infections of Leishmania localised constantly in the peritoneum as the result of intraperitoneal, intra-hepatic, and even subcutaneous injections of an emulsion of organs of a dog infected with leishmaniosis. But by means of cultures infection was not obtained in the mouse, even after injections repeated many times and with increased doses. This was established by Delanoë, who has thrown light on the mechanism of the natural immunity possessed by the mouse against the cultures, showing that in the peritoneum a very active phagocytosis of the flagellate forms takes place, so that after a short time they are broken up and completely destroyed.

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[1] It is well to point out that Franchini, when he uses the name L. donovani, means (at least so I believe) the Leishmania which is the specific agent of kala-azar in Italy.

Continuing previous researches left incomplete, I have again undertaken inoculations of guinea-pigs and white rats with cultures, having at my disposition besides the strain obtained by myself from a young patient, Rocca V—, of Bovalino Calabro, other strains also which I owe to the kindness of Mesnil and Jemma. While the cultures of the Pasteur Institute of Paris, originating from Tunis, had been kept alive for some years by successive transplantations, mine and those of Jemma were very recent and still in the first subcultures. The cultures used were at the height of their development, some in the first days of the subculture (3, 5 or 7 days), others older (10, 15 or 20 days) and others very old (1, 2 or 3 months). The object was to inject all the various forms which have been described in the cultures of Leishmania. The quantity injected was almost always 2 c.c., sometimes 4 c.c., for animals of medium size, guinea-pigs weighing about 300 gr. and young rats. In some cases 1 c.c. of the culture was injected, to try the effect of injecting the animals once, twice, thrice or four times. Twenty guinea-pigs and 10 rats were inoculated.

The method of injection was for the most part intra-peritoneal, and only in a few cases was the material of the culture injected under the skin or directly into the current of the circulation, by the vein of the tail of the rats or by means of heart-puncture—a method which seems to me preferable since it does not present excessive difficulties of technique.

Some of the animals died spontaneously, two guinea-pigs and two rats, in the course of the investigations, as happens often with these animals in the laboratory from causes extraneous to the experiments, and of these I made a rigorous examination in search of the Leishmanias; only one rat and one guinea-pig were lost without examination, and of these, of course, I have taken no account. The others were killed by chloroform at various intervals of time from the first injection, from an hour and a quarter to ninety-three days after, and the autopsy was performed immediately. Both the guinea-pigs and the rats showed no signs of loss of flesh; some of them were even increased in weight. While they were still living, with a glass pipette having one end drawn out into a capillary tube I took from the peritoneal cavity a drop of yellowish liquid, slightly turbid, containing, as we shall see further on, numerous leucocytes, chiefly mononuclears.

The subsequent autopsy revealed nothing noteworthy; the internal organs, spleen, liver, kidneys, bone-marrow and lungs were not increased in volume or changed in appearance or in any way different from those of healthy animals. Of these organs I made smears which I fixed afterwards either in methyl alcohol or with osmic acid vapour and absolute alcohol and stained with Giemsa's stain. The most careful examination has never enabled me to discover a Leishmania, either in its usual form or in the flagellate leptomonad or other type, even when the autopsy followed close upon the inoculation of the cultures into the peritoneum.

Both of the blood and of the internal organs I have tried in each animal numerous cultures in blood-agar (method of Novy and Nicolle), and I have never obtained development of Leishmanias; the culture-tubes remained perfectly sterile, save for some rare exceptions (see below).

I think, therefore, that I am able to establish the point that guinea-pigs and rats possess a natural immunity against the cultural forms of the Leishmania of the Mediterranean basin, alike whether the strains are recently isolated or of long standing and whether the cultures are new or old subcultures.

The case of the infection of the guinea-pig obtained by Franchini with cultures remains, therefore, unique and must at least represent a somewhat rare event. It was a case perhaps of a single animal extraordinarily and exceptionally receptive. Exceptional at least are the observations which Franchini describes. He found parasites in the peripheral blood and free in the plasma, and similarly all the forms of Leishmania found in the liver, spleen and bone-marrow were extra-cellular. It is, however, usual in Leishmanial infections, by general agreement of all observers, for the parasites to be always or nearly always contained in the protoplasm of the large mononuclears, and it has not yet been shown that they can occur free outside this their natural situation. Unfortunately the drawings which accompany the work of Franchini are so obscure and so little demonstrative that they do not permit one to judge whether one is dealing with Leishmania, the more so since the methods employed by the author are certainly not those the best adapted to obtain elective staining, but are even scarcely sufficient for diagnostic purposes, especially when the parasites, as in this case, are rather scarce.

In order to study rather more closely the mechanism of the natural immunity possessed by guinea-pigs and white rats, and at the same time to avoid any objection to the facts observed by me, I have carried out a double series of investigations, morphological and cultural, with the object of following step by step the fate of the flagellated forms in the organism of the experimental animals.

Having injected into the peritoneum 2 c.c. and sometimes 4 c.c. of a culture very rich in flagellated forms, I drew off every five minutes some peritoneal fluid with a sterile capillary glass tube and examined it fresh with the microscope, making at the same time some preparations for staining. The fact is quickly observed that the parasites undergo absorption rapidly by the leucocytes in the peritoneal cavity.

Prior, however, to describing the phenomenon in greater detail some points of technique may advantageously be dealt with. I have performed the experiment on a sufficiently large number of animals, guinea-pigs and rats, in such a way as to be able to perform, in each animal, only one or two punctures of the peritoneum, with the object of avoiding those alterations which are produced by the simple fact of operative lesion. With regard to this I can confirm the observation of Delanoë for trypanosomes. When the puncture of the peritoneum is repeated several times, alteration and death of the Leishmanias take place by the probable extrusion of trypanolytic substances from the leucocytes into the plasma, while if the peritoneum be not punctured the flagellates that have remained free are preserved living and with normal structure, and in this condition are engulfed by the leucocytes.

Of the liquid extracted from the peritoneal cavity I have made preparations in the fresh condition and smears on slides which I have fixed while still wet in vapour of osmic acid for five seconds, and afterwards, when dry, in absolute alcohol for a quarter of an hour—a method of technique which also gives very good results for staining the flagellates in the cultures. The smears should be very thin, and it is often useful to follow the staining by very short differentiation in the solution of tannin according to Unna.

I have made examinations of the peritoneal liquid every five minutes after the injection up to two hours and then every half hour up to four hours, repeating the experiment in a fairly numerous series of animals. In the fresh state it is seen in the first few minutes after the injection of the culture that the number of the Leishmanias, truly enormous in 2–4 c.c. of a rich culture, is already to some extent diminished, and not by the fact of the greater dilution alone; very many flagellates are still free and very many are mobile, perhaps even more than in the cultural liquid, but it is not difficult to come upon others adhering or united to leucocytes, either by the posterior end or by the flagellum, preserving, however, in all cases a certain mobility. When they are completely engulfed in the leucocytes they are seen in its protoplasm as rounded bodies, but at this point it is easier to recognise them and to follow their modifications in the stained preparations. In the fresh state I have found in the guinea-pig some Leishmanias free and mobile up to an hour or an hour and a half after the injection of 2 c.c. of culture into animals of 250 to 300 grammes in weight. In the rat I did not succeed in seeing free forms after an hour and a quarter.

In the stained preparations these various phenomena are very evident, and it is possible to follow step by step the various phases of alteration to which the Leishmanias are subjected.

One fact which deserves special emphasis is that the parasites are found always in the large mononuclears and in them alone, that is to say in just those elements which contain them in kala-azar and oriental sore. In the rare cases in which a true polynuclear is found apparently containing a Leishmania, the possibility cannot be excluded that it is simply superposed rather than engulfed. In the protoplasm of the mononuclears they become more rounded in form, preserving sometimes all their constituent parts and traces of the flagellum. Thus it is possible in some parts of the preparations to have the impression that it is a case of simple modification of the form of the Leishmanias and that they have found in the mononuclears a habitat adapted to them; but for the most part they present obvious alterations. The protoplasm becomes clear or turbid, the contours of the parasite disappear, the flagellum becomes thickened and breaks up; sometimes the trophonucleus or the kinetonucleus is wanting, or these bodies lose their sharp contours and their special characteristics and are reduced to shapeless masses of chromatin.

After fifteen to twenty minutes very many mononuclears are observed containing Leishmanias profoundly altered, represented after twenty to thirty minutes by remains of nuclei near which a portion of the flagellum is to be noted. The profound modifications of the phagocyted parasites continue, and after two to three hours there are found only chromatinic granules with masses of protoplasm stained intensely in reddish violet, representing the last vestiges of the destroyed Leishmanias.

Even after thirty minutes up to an hour the first stages of the phagocytosis are still to be observed; mononuclears which contain forms greatly altered show others in process of being engulfed, while other flagellates still quite intact remain free and perfectly mobile in the serum, like that shown in Fig. 7, twenty-five minutes after the injection.

I have also made, as already stated, preparations of the peritoneal exudation of the animals sacrificed at various periods up to ninety-three days after the injection of the cultures and I have never seen Leishmanias or remains of them in the mononuclears, although I have directed special attention to this point, because Laveran and Mesnil have observed the peritoneal infection up to fifty-nine days after the injection of emulsion of organs of a dog infected with experimental leishmaniosis and have obtained subcultures of this fluid.

Contemporaneously with the examination of the peritoneal fluid I have repeatedly made an examination of the peripheral blood, and, in animals sacrificed, I have made smears of the various organs; in spite of the most patient investigation I have never met with any form of Leishmania or any flagellate parasite in my numerous preparations.

The facts described permit me to assert that we are dealing with a process of phagocytosis pure and simple; it cannot be admitted that the flagellate leptomonad forms are transformed into Leishmania-forms and as such continue to live in the mononuclears, producing a slight infection localised in the peritoneum, of the type of that obtained by Laveran and Pettit, because we are witnesses here of a gradual alteration and destruction of the parasites by a process which leads to the final disruption of all remains of the injected Protozoa. It is scarcely necessary to add that in the forms engulfed every sign is lacking of a process of multiplication. Moreover the results of attempts to make cultures confirm fully this interpretation, and give a proof of it which I think can be regarded as absolute. In every animal killed with chloroform I have made cultures in blood-agar according to Novy-Nicolle of blood taken aseptically from the heart, spleen, liver, bone-marrow, and in some cases of the peritoneal fluid. The results were constantly negative except with the peritoneal fluid a short time after the injection of the culture, when it is still possible to find by microscopic examination some Leishmanias living and mobile. Thus after an hour and a quarter in one rat I was able to obtain a new culture from the peritoneum, while after two hours the result has always been negative.

In complete accord with the result of the histological examination I have never obtained development of flagellates in a culture from the blood or from the internal organs, not even by injecting into the peritoneal cavity or directly into the circulation 4 c.c. of cultural fluid very rich in Leishmanias. These flagellate Protozoa would not, therefore, pass beyond the peritoneal barrier in the guinea-pig and the rat.

I have stated above that I have injected the cultures of Leishmania directly into the blood in some animals. Although my investigations may not be sufficiently numerous, being limited to one guinea-pig and one rat alone, I have noted in the blood immediately after the injection a marked leucocytosis with prevalence of mononuclears, which reached its maximum degree after five to ten minutes, was maintained for half an hour, and then diminished gradually. In these cases also I was never able, even soon after the injection, to find flagellate Leishmanias in the blood, nor have I seen phagocyted forms. The animal has not contracted any infection.

SUMMARY.

Guinea-pigs and rats possess natural immunity to a high degree against the Leishmania of the Mediterranean basin in the flagellate stage in cultures on blood-agar (method of Novy-McNeal-Nicolle).

This immunity is exclusively of phagocytic nature. The Protozoa injected into the peritoneum become rapidly en-

gulfed by the leucocytes, the mononuclears exclusively, and undergo gradual and progressive alterations, ending in their complete destruction, so that by the end of an hour or an hour and a half after the injection of 2 c.c. of a culture in full development, free flagellates are no longer to be found in the serum, and after two or three hours even the last vestiges of them are almost destroyed in the protoplasm of the phagocytes.

The Protozoa do not pass beyond the barrier of the peritoneum and do not find their way in this manner into the blood or the internal organs.

These data of observation receive full confirmation in the results of the cultures. It is possible, in fact, to obtain subcultures from the peritoneal fluid up to about one hour after the injection of 2 c.c. of liquid of condensation very rich in Leishmanias, but not beyond this period, nor are subcultures obtained from the blood or organs of the animals in experiment.

I desire to express my gratitude to Dr. Martin for having received me with so much kindness and hospitality at the Lister Institute of Preventive Medicine, London, of which he is the Director, and for having provided me liberally with the means of research; I also thank Dr. Bayon warmly for assisting me in every way during my stay in this Institute.

Lister Institute of Preventive Medicine,
London,
May 25th, 1912.

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DESCRIPTION OF PLATE 21

(From smears of peritoneal fluid),

Illustrating Dr. Arrigo Visentini's paper on " The Transmission of Leishmaniosis by means of Cultures, and the Mechanism of the Natural Immunity in Rats and Guinea-pigs."

[The figures are drawn with the camera lucida at a magnification of 2000 diameters.]

Fig. 1.—Five minutes after the injection of a culture of Leishmania, showing one parasite absorbed, and another in process of absorption, by a mononuclear leucocyte.

Fig. 2.—Ten minutes after injection; a leptomonad form absorbed, another held fast by its flagellum.

Fig. 3.—Thirty minutes after injection; a mononuclear containing three parasites, in process of destruction, and in the act of absorbing a fourth.

Figs. 4 and 5.—Fifteen minutes after injection; mononuclears containing Leishmanias in process of destruction.

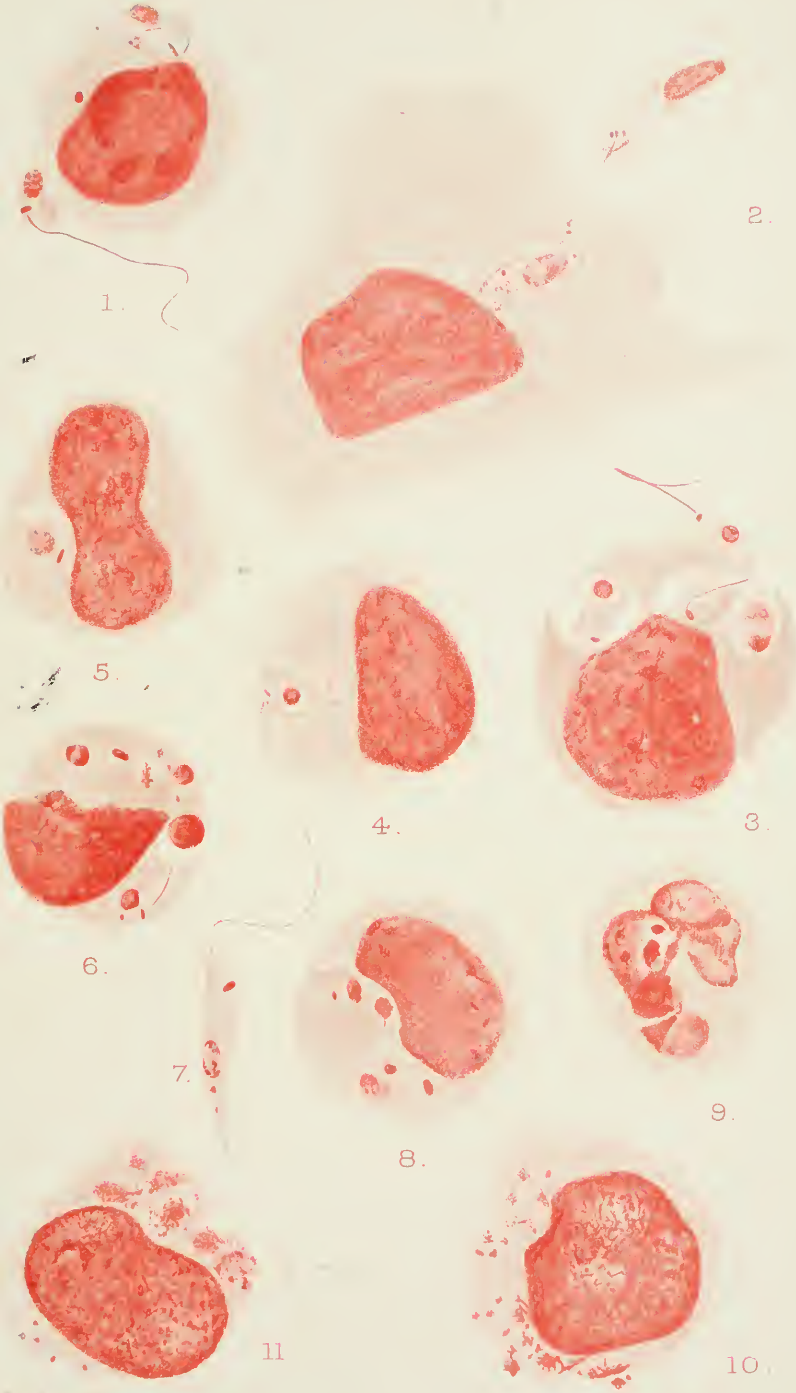
Fig. 6.—Twenty minutes after injection; mononuclear containing the remains of several parasites.

Fig. 7.—Free Leptomonas-form twenty-five minutes after injection.

Figs. 8 and 9.—One hour and a quarter after injection; a mononuclear containing remains of several parasites and a polymorphonuclear apparently containing (?) a parasite.

Fig. 10.—Two hours after injection; mononuclear containing the last vestiges of several parasites.

Fig. 11.—Three hours after injection; as the last.



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PHAGOCYTOSIS OF LEISHMANIA

N° 45

Notes on Sporozoa. Nos. II, III, and IV.[1]

By

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Lister Institute of Preventive Medicine, London.

With Plates 9 and 10.

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PREFACE.

While working at Rovigno during April and May, 1909, I examined a few of the common wall-lizards (Lacerta muralis), which occurred abundantly on the neighbouring islet of Figarola, as in the time when Prowazek studied the intestinal flagellates of this reptile (**26**). My object was really to see if a Trypanosome also occurred in it, Prowazek not having stated whether he examined the blood for that purpose or not; but I did not succeed in finding any Trypanosomes. In the blood-smears made from two individuals, however, a Hæmogregarine was found to be fairly plentiful. This Hæmogregarine is the same as that first described by Danilewsky (**6**) under the name Hæmogregarina lacertæ, and again later by Labbé (**13**), who placed it in a distinct genus, Karyolysus. I was too much occupied with other work at the time to undertake a study of this parasite, so that I only made a few smears and cover-slip preparations from the blood.

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[1] For the first of these Notes ("On Klossiella muris, Smith and Johnson"), vide 'Quart. Journ. Micr. Sci.' vol. 48, 1904, p. 153.

Having an opportunity recently, I thought it might prove worth while to give some attention to these preparations, particularly to those stained by iron-hæmatoxylin, because—so far as I am aware—no observations have been made up to the present upon the nuclear structure of Karyolysus, as it is seen when the parasite is fixed and stained by the best cytological methods. My idea was, principally, to compare the nucleus of this Hæmogregarine with that of the piscine form (Hæmogregarina rovignensis) from Trigla lineata, an account of which has been given by Minchin and Woodcock (**20**). I had not long examined my preparations, however, before observing that a remarkable agreement was apparent between the nuclear condition of Karyolysus at a certain period of the life-cycle and that of a particular Coccidian in the corresponding phase. A study of the different forms of individual present in my smears has led me to the conclusion that they all belong to one species of parasite. This result has an important bearing, in my opinion, upon the question of the distinctness of many of the so-called species of Lacertilian Hæmogregarine which have been described, as I hope to show below. Lastly, the observation of the occurrence of a prominent karyosome, whose behaviour agrees closely with that of the characteristic coccidian karyosome, induced me to study again, from this point of view, the nuclear condition present in Leucocytozoon and Halteridium, as it is found in these parasites when fixed and stained in a similar manner.

II. OBSERVATIONS ON KARYOLYSUS LACERTÆ (DANIL.), TOGETHER WITH REMARKS UPON THE SPECIFICITY OF THE HÆMOGREGARINES OF LIZARDS.

I will first give an account of Karyolysus as it occurs in my preparations. With two or three exceptions, all the individuals observed are intra-cellular. In the blood of both the infected lizards the great majority of the parasites occur under one of two different aspects, which might lead one, at first sight, to conclude that two distinct species were concerned; but after a careful comparison of many individuals of both kinds, no doubt is left in my mind that they represent respectively early young phases and rather later, older forms of one and the same parasite. The two types of form (as they may be designated for the present) are distinguished chiefly by the position and character of the nucleus, which I will consider in detail presently; the latter feature can only be studied properly in preparations made by the "wet" method. I will merely say here that in the first type of individual the nucleus is situated more or less about the middle of the body (Pl. 9, figs. 2–8, 19–29), whereas in the other type it is close to one end (figs. 9–18, 30–40).

As regards their general appearance, both kinds of individual are usually bean-like in shape, the younger parasites being more slender and now and then slightly crescentic, the older ones broader and stouter. The individuals of both types vary somewhat in size, the former being, as might be expected, slightly shorter on the whole and distinctly narrower than the latter; but a few forms which possess the nuclear characters of the first type are met with, which are approximately as large as others possessing a nuclear arrangement of the second type. The dimensions of the younger forms, as seen on "wet" films, vary from 8 μ by $2\frac{1}{8}$ μ (fig. 22) to $9\frac{1}{2}$ μ by $2\frac{1}{2}$ μ (fig. 26); those of the older individuals from 9 μ by $2\frac{1}{4}$ μ (fig. 39) up to $11\frac{1}{2}$ μ by 3 μ (fig. 33). On "dry" smears the larger parasites are probably rather flattened out; the extreme limits of variation in size (of either kind of individual) noticed are from 11 μ by $2\frac{1}{2}$ μ (fig. 2) up to 13 μ by $4\frac{1}{2}$ μ (fig. 17). The largest bean-shaped individuals, however, such as those of figs. 14–17, have undoubtedly acquired that appearance secondarily, by the lateral fusion of the two arms of a U-shaped form, the U-shaped form resulting in the first place from the further growth and extension of the body-cytoplasm of a smaller individual. I have found different stages of the process in my preparations. The development of the U-form takes place only, so far as I have observed, in those parasites in which the nuclear position is that of the second type mentioned above. It begins by the formation of a small outgrowth at one end of the body, which is at once curved back and so extends backwards close along one side of the body (figs. 10, 36, 40). This outgrowth may arise either at the nuclear end of the parasite or at the opposite one, more usually, I think, at the latter. As it grows this process gradually forms one arm of the U, and at length the two arms become more or less equal (figs. 14, 15). Ultimately the two arms unite and a stout bean-shaped form results.

In nearly all of the individuals observed in "wet" preparations, of whichever type they may be, immediately surrounding the body of the parasite is a distinct space, which in some cases is very marked (cf. figs. 20, 22, 24–40). This space is probably due to the greater contraction of the parasite, as a result of the technique, than of the cytoplasm of the red blood-corpuscle enclosing it, thus causing a shrinkage of the former away from the latter. In ordinary "dry" smears, stained with Giemsa, this space is also often seen, though not so regularly as in the other preparations (cf. figs. 5–8, 12). In the case of the smaller parasites there is probably no definite membrane or envelope bordering the space on its outer side, distinct, that is to say, from the inner margin of the cytoplasm of the corpuscle (cf. for instance fig. 19, where the young Hæmogregarine has obviously just entered the host-cell). In the older (larger) forms, however, there is certainly a definite envelope present, constituting a delicate but firm capsule around the parasite (cf. especially figs. 37–40). In the case of two of the parasites figured it will be noticed there is no sign whatever of the cytoplasm of the blood-corpuscle; the reason for this will be mentioned shortly (pp. 177, 179). Hence the capsule surrounding the parasite is very conspicuous. In many cases where the cytoplasm of the host-cell is still apparent the delicate capsule does not stand out in such a marked manner, but its presence is clearly indicated, in my opinion, by the following consideration. Many of the older forms, those, i.e., of the second type, in which the nucleus is situated near one end, appear very dark in "wet" preparations, being stained diffusely and more or less uniformly, so that it is very difficult to distinguish the nucleus. This appearance is really owing to the stain deposited inside the capsule or envelope not having been sufficiently extracted subsequently, the differentiating agent not having had time to penetrate properly inside the capsule, thus leaving the parasite overloaded with stain. I have never found this state of things, it should be noted, in the younger parasites, with the nucleus still near the middle; hence the capsule does not appear to be formed during the early phase. This capsule or envelope present in certain forms of Karolysus appears to be very similar to that described in the case of small forms (young schizonts) of Hæmogregarina triglæ by Minchin and Woodcock (20). In the case of Karyolysus, however, I am inclined to think that the capsule is rather a definite envelope formed by the parasite than merely a sheath or altered layer of the cytoplasm of the blood-corpuscle (cytocyst), as we regarded it at the time in H. triglæ; its persistence and distinctness in such individuals as those drawn in figs. 37 and 38 supports this view.[1]

Considering now the nuclear structure in detail (as it is seen in "wet" preparations, stained with iron-hæmatoxylin), in the first type of individual, where the nucleus is situated near the centre of the parasite, the most striking feature is the very frequent occurrence of a conspicuous, deeply staining body, which is closely associated with the nucleus, lying at one side of it, contiguous to, but not actually forming part of, the general nuclear substance (figs. 19, 21–25). This latter consists, as in other Hæmogregarines, of a network containing small but fairly prominent grains of chromatin, most of which are usually disposed near the periphery. The limit or border of the nucleus is well-defined, but I am a little doubtful whether it can be regarded as constituting a true nuclear membrane. In some cases there are two of the above-mentioned conspicuous bodies, approximately at opposite sides of the nucleus (figs. 20, 26); these are generally unequal in size, neither being as a rule so large as when there is only one. Frequently these elements are seen to be surrounded by a very clear zone or halo (figs. 19, 20 and 25).

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[1] The mode of origin of the capsule may be really the same, of course, in H. triglæ also.

In the other type of individual there is usually no such large, deeply staining element associated with the nucleus (figs. 30, 32–36, 38–40). This contains fairly uniform grains of chromatin, which, on the whole, are distinctly more prominent and stain rather more deeply than those in the nucleus of the first type; now and again one of these grains is seen to be somewhat larger than the rest. Neverthess, in a few instances, parasites belonging to this second type of form, with the nucleus near one extremity, do also show a large, deep-staining body in close association with the nucleus (figs. 31, 37), which is quite comparable to, or at least represents, that seen in the case of individuals of the other type. Much more frequently, however (though not always), in place of this element close to the nucleus there is noticeable a body lying at or near the surface of the protoplasm of the parasite, usually about the middle of its length (figs. 32–36). This structure may be nearly as large as that just described, but it is generally smaller, and may be very inconspicuous (fig. 36); where it is large it stains fairly intensely, but it is never so dark and black-looking as in the other cases, and, moreover, it has a much duller appearance and not such a well-defined outline.

I propose to leave for the moment the question of the significance of these bodies. It may be added that in Giemsa-stained smears on the other hand, in which the nucleus of the parasites generally appears to consist of large, irregular or ill defined masses of chromatic substance, it is only seldom possible to distinguish a more deeply staining element at one side, which probably represents the characteristic element above described (cf. fig. 8).

The effects of the parasite on the host-cell are very pronounced and characteristic, as is well known to be the case, of course, in Karyolysus. The gradual alteration of the red blood-corpuscle and its appearance when infected by the different forms of parasite merit description, however, since this change is of great importance and assistance both in determining the relation to each other of the two chief types I have described, and also in connection with the question of the various species of Hæmogregarine (Karyolysus) said to occur in this lizard. The earliest change in the appearance of the host-cell which I have noticed is drawn in fig. 2 (from a Giemsa smear). The parasite infecting this corpuscle is one of the smallest observed, and has the nucleus centrally placed. Comparing the host-cell in this case with an ordinary uninfected red blood-corpuscle, its nucleus is found to be already distinctly larger, i.e. hypertrophied, but still oval in shape and not much elongated. The cytoplasm of the corpuscle is also slightly hypertrophied, but it is still stained to about the same degree and shade of colour as in an uninfected cell. This is, however, almost the only instance I have noticed where the cytoplasm appears stained similarly to what is the case in an uninfected cell. It is remarkable how quickly the presence of a Karyolysus-individual in a corpuscle produces some effect on the cytoplasm which results in a complete alteration of its staining properties. In nearly all the corpuscles infected with this Hæmogregarine, the cytoplasm has either taken up the stain only slightly, being faintly coloured, or else is very pale, practically unstained, so that it is often a matter of extreme difficulty to discern it at all. This is especially the case in wet preparations, stained by iron-hæmatoxylin; and in this respect Karyolysus differs markedly from certain other intra-cellular parasites of red blood-cells, of which I have preparations stained in a similar manner. For example, in Hæmogregarina triglæ (cf.

the figures of Minchin and Woodcock, loc. cit.) and again in Halteridium noctuæ (cf. below), the cytoplasm of an infected corpuscle is usually stained deeply, like that of an uninfected one, even where, as in the former case, there is a certain amount of hypertrophy; in these, the parasite appears as a clear space, almost vacuole-like, surrounded by the dark cytoplasm. In Karyolysus the appearance is quite different. Figs. 20–22, 24–26 represent early stages in an infection as early as, or slightly later, than that of fig. 2, from a Giemsa smear. The nucleus of the corpuscle is either oval or beginning to elongate. In such cases the cytoplasm can still be made out, but it never appears any darker than is indicated in Figs. 20, 22, 24. The nucleus also retains the stain much less intensely than in an uninfected cell stained by the same method (c.f. fig. 23), the actual masses and grains of chromatin standing out sharply from the finely granular or reticular ground substance. The host-cell nucleus, it will be seen, is at once displaced by the parasite, and pushed to one of the longer sides of the corpuscle.

From being oval or slightly extended, the host-cell nucleus gradually becomes considerably elongated and greatly narrowed, i. e. compressed (figs. 32–40); all stages in this transition can be found, the real change in shape being best realised, of course, in preparations stained by iron-hæmatoxylin. In most cases the corpuscle-nucleus, in its final condition, appears like a slightly crescentic band, which is closely apposed to the parasite (or rather to its envelope) and follows its contour, curving round somewhat at either or both ends; this portion of the cell-nucleus is generally a little broader, i. e. less compressed than the rest, giving the whole nucleus the appearance of a bent club or halter, as the case may be.[1] In all these instances the axis of extension of the host-cell nucleus is approximately parallel to the length of the parasite. Now and again, however, where the corpuscle-nucleus has either not been quite parallel to the longer axis of the cell to start with, or else has become twisted round somewhat by the entry of the parasite, the longer axis of the Karyolysus is more or less oblique to that of the host-cell nucleus, the one lying, as it were, across the other; in these cases, fission of the host-cell nucleus into two or more portions nearly always results (figs. 29, 11, 30). An important point must be mentioned regarding the appearance which one of these nuclei, in its final condition of hypertrophy, may occasionally present on a Giemsa smear, since it affords, I consider, another example of how the over-staining tendency of this stain may mislead and cause erroneous interpretations. In a few cases a mass of staining substance is seen, fitting like a cap round one end of the parasite, or there may be such a mass round both ends (figs. 16–18). These masses stain similarly to the nucleus of the host-cell, lying at one side of the parasite, and in fact may be distinctly connected with this and manifestly portions of it; it may happen, however, that such a mass appears almost or entirely separate from the nucleus, especially in flattened-out parts of the smear. Nevertheless there can be no doubt that these caps of staining substance represent also in such cases merely the wider, club-shaped end-portions of a crescentic host-cell nucleus, as above described, only here they are greatly overloaded with stain. These "caps," it is important to note, are distinctly on the outer side of the capsule enveloping the Hæmogregarine.

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[1] The resemblance between this hypertrophied nucleus and that of the spindle-shaped host-cell infected by Leucocytozoon is often striking (cf. figs. 11, 12, 18 and 19, Pl. 10).

As indicated above, the cytoplasm of the infected corpuscle becomes ultimately so colourless that it is quite impossible to discern it (cf. figs. 37, 38 from wet preparations and fig. 17 from a Giemsa smear); in these cases it cannot be said whether it is still present or not.

The two forms of the parasite can now be considered in relation to the particular degree of alteration shown by the host-cells respectively infected by them. As a rule, in corpusles which are in the earlier stages of alteration, with the nucleus still oval or only beginning to elongate, the parasites are of the first type described, with the nucleus central and having the conspicuous, deeply staining body in close association with it (figs. 20–26). On the other hand parasites of the second type, with the nucleus near one end of the body, occur nearly always in corpuscles in which the alteration is far advanced, the cytoplasm being, at the best, only with difficulty discernible and the nucleus greatly elongated and narrowed (figs. 32–40). As is only to be expected, however, occasional exceptions to the above regular conditions are to be met with. Thus, an individual may be found, having its nuclear arrangement of the first type, which has already caused considerable elongation and alteration of the host-cell nucleus (figs. 8, 28, 29); conversely, a parasite may have acquired the second type of nuclear condition before the corpuscle-nucleus has become very elongated and narrow (figs. 10, 31). It may be regarded as practically certain, therefore, that the second type of individual is a rather later or older phase in the development of the first type of parasite. In addition to the evidence afforded by the various stages in the alteration of the infected corpuscles, this conclusion is also supported by the following points. Parasites of the second type are on the whole distinctly larger, that is to say, they have more bulk than those belonging to the first category; further, the only individuals seen free (fig. 21), or which have manifestly only recently entered a corpuscle (figs. 19, 26), have the first type of nuclear arrangement.[1]

It is not difficult, I think, from a careful comparison of different individuals, to form a fairly accurate idea of the manner in which the change in nuclear position and character is brought about; and for this purpose it is necessary to study the behaviour, in relation to the nucleus, of the characteristic deep-staining body which is associated with the latter in the young forms of the Hæmogregarine. In the earliest phase this body, which from now onwards I will designate according to its true significance, namely, as a karyosome, is single and relatively large; it is situated at one side of the general nuclear substance, apparently extra-nuclear. This large karyosome next undergoes unequal division. The process takes place in a particular manner, which is neither amitotic nor yet a well-defined mitosis. This method of division has been usefully distinguished by Nägler (**22**) as "promitosis."

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[1] I have never observed any individuals of the larger, older type free—that is to say, which could have been liberated from a corpuscle, whether with or without the enveloping capsule.

It may be as well to indicate, first of all, what is meant by promitosis. Its characteristic feature is that the division is initiated and carried out by means of an internal division-centre, which itself first divides, the two resulting daughter-centres then passsing away from each other, but remaining connected by a distinct fibril or axial thread, the "centrodesmose." The term "promitosis" was originally applied by Nägler to nuclear division taking place in this manner, the intra-nuclear division-centre being a "nucleo-centrosome" or a karyosome. Where the karyosome plays this part, however, the true division-centre—certainly in most cases, and perhaps always—is an intra-karyosomatic centrosome or centriole, which initiates the process, although, owing to the intensity with which the karyosome usually stains, the centriole itself can rarely be distinguished, its presence being often only actually discernible at some other period in the development (cf. below, p. 182). Fortunately, however, the axial fibril or centrodesmose connecting the two separated daughter-centrioles persists often for a long time, even after the division of the karyosomatic or nuclear material is completed; hence it is just this stage of the division-process which is most likely to be observed. Therefore, where two nuclei (or karyosomes) are seen still connected by a definite centrodesmose, it may be safely concluded that the division has been brought about by an internal division-centre (centriole), in a promitotic manner. It only remains to say that I consider the term "promitotic division" can also be applied very suitably to the division of a karyosome, where this occurs unaccompanied by, or independently of, the division of the nucleus itself; Jollos (**12**) has already used the term in this connection.

It is undoubtedly in the above-described promitotic manner that the unequal division of the karyosome takes place in the young forms of Karyolysus; for I have found two or three examples which show very clearly the still persistent centrodesmose between the two halves (fig. 20). This fibril stretches apparently across the general nuclear mass; but it may really lie outside it, i. e. above or below; I do not feel sure upon the point. The smaller daughter-karyosome resulting from the division always comes to lie at the opposite side of the nucleus to the other, larger one (figs. 24, 26). This smaller, secondary karyosome, however, soon becomes incorporated with the general nuclear material; either it is distinguishable as a rather larger and more prominent grain, or else, probably having undergone further subdivision, it can be no longer distinguished from the rest of the chromatic substance. Now and again, it may be mentioned, in such a nucleus a small, but sharp and well-defined granule is seen in the centre; this may very likely be the centriole (fig. 40). The nucleus has by this time changed its position and passed to one end of the body of the parasite. In the majority, if not in most cases, it leaves behind it the larger half of the karyosome, which resulted, i. e., from the original promitotic division; this remains near the middle of the body, the nucleus simply moving away from it. Why this change in the nuclear position occurs I cannot say; it might be supposed, perhaps, that it had some connection with the commencing development of the U-form of the parasite, but the bending of the cytoplasm sometimes takes place at the end opposite to that to which the nucleus travels. Whatever the reason, this movement occurs, I should say, very rapidly, for I have not succeeded in finding an individual which shows the nucleus caught in the act, as it were, halfway between the end of the body and the stationary karyosome. This latter element thus left behind takes no further share in the nuclear development, and appears to be entirely discarded. As already indicated, it alters considerably in staining properties and in definiteness of outline; it gradually becomes smaller and smaller, being perhaps partially used up by the cytoplasm, and ultimately its remains are seen at the surface of the body (figs. 34, 35). Often, however, no trace of this body is left (figs. 30, 38 and 39). On the other hand, occasionally this large karyosome seems to persist and to change its position with the nucleus (figs. 31, 37). In such cases it lies nearest to the end of the body, between this and the nucleus, having been pushed along as it were by the nucleus, instead of being left behind. Possibly the reason for this occasional persistence of the large karyosome as a separate element in close association with the nucleus, after the latter has changed its position, may be that the karyosome has not yet undergone the above-described division—a division which may be necessary in order to eliminate an unrequired portion of the karyosomatic material before the remainder is added to the nuclear substance. I have no evidence as to the further behaviour of the karyosome in these cases.

I can now summarise the general course of the early development in Karyolysus, so far as I was able to ascertain it. The different types of form observed are phases of one parasite. A small individual, such as that of fig. 21, penetrates a red blood-corpuscle (fig. 19) and begins to grow. As the parasite grows, changes in the nuclear constitution and position take place. At about the same time a definite envelope or capsule is formed around the parasite, inside which the latter tends to acquire, by bending up, a characteristic U-shape, and ultimately becomes stout and bean-like. The presence of the Hæmogregarine causes very great changes in the appearance of the host-cell, hypertrophy and pronounced alteration in the shape of the nucleus, sometimes its fission; further, the cytoplasm, or what remains of it, loses almost entirely its staining properties and becomes extremely difficult to see in the preparations.

From a comparison with Reichenow's valuable and detailed account (**27**) of the development of Hæmogregarina stepanovi of the tortoise, there can be little doubt that the forms I have described of Karyolysus are phases in the development of the schizont, i. e. the form which undergoes schizogony or endogenous multiplication. A point in regard to which I cannot be certain is whether these young schizonts are the first to be developed, as the result of a fresh infection, or whether the infection is of some standing and these forms have been produced by a prior schizogony; in other words, whether the small, free individuals are developing sporozoites or merozoites. The only indication bearing upon the point which I can note is that the nuclear constitution of the young individuals, showing a distinct excentric karyosome, agrees markedly with the nuclear condition found in the developing merozoites of certain Coccidia and differs from that present in the sporozoites. I intend to discuss this agreement more particularly later, and will merely say here that this evidence favours the view that the schizonts which we have been considering are developing from merozoites.

The Question of the Specificity of the Hæmogregarines of Lizards.

I wish now to discuss the question of the specificity or true distinctness of certain of the many alleged species of Hæmogregarine (Karyolysus) which have been described from Lacerta spp., chiefly from the common European species agilis, muralis and viridis; my object is to show that some, at any rate, of these new species are almost certainly nothing more than different forms or phases of one and the same parasite, Karyolysus lacertæ. As I have had occasion to point out more than once in previous papers, the custom is far too prevalent of regarding any difference in appearance, or variation in size or form, observed in individuals of a certain genus of blood-parasites (and particularly in the case of Trypanosomes and Hæmogregarines), as indicating a distinct species, even though this "new species" occurs in a host in which a parasite of the same kind is already known. Often the view which is at least quite as probable, and in many instances more so, namely that the forms in question are phases in the life-history of one and the same species of parasite, receives no consideration, and no attempt is made to connect the various types by means of intermediate stages. I am glad to see that Laveran and Pettit, in a recent note (**15**), also express a similar opinion, and comment upon the confusion liable to be caused by creating new species in the above casual manner.

To begin with the original description of Hæmogregarines from lizards, i. e. the account given by Danilewsky (**6**), this author observed various forms of the parasites in L. agilis and viridis. Making all allowance for the fact that Danilewsky's description and figures are mostly based on observations on the living parasites in the drawn blood,[1] and also for the primitive character of microscopical technique in those days, it seems probable nevertheless that this author was actually dealing with more than one species. Here, as in other cases (for instance, his memoirs on Trypanosomes), it is extremely difficult to gather what Danilewsky intended to mean by his grouping of different forms and the nomenclature he applied to them. He distinguishes three intra-cellular types (A, B and C), which he regards as having a genetic connection ("lien génétique") with one another. To these, collectively, he gives the name Hæmogregarina lacertæ; but immediately afterwards the second type (B) is termed Drepanidium lacertarum, because it is smaller and younger; while in another part of the memoir the third form (C) is called Hæmocytozoon clavatum! The last type is generally considered to be distinct; this is, I think, most likely, particularly since it does not produce, to judge from Danilewsky's account, hypertrophy of the blood-corpuscle and alteration of its nucleus; in other words, it is apparently not a karyolysing form at all. The small type (B) may be a young phase of (A); more than this cannot be said. At any rate it is to the first described parasite (type A) that the specific name lacertæ really belongs. Comparing the different forms of the Hæmogregarine I have described above, from L. muralis, with Danilewsky's description and figures of H. lacertæ, it is perfectly clear that the parasite is the same species in both cases, and, moreover, in the same period of development; some of Danilewsky's figures are of young forms, with the nucleus near the middle and the host-cell only slightly altered; others are of the older phase, with the nucleus at one end and the nucleus of the corpuscle completely karyolysed.

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[1] While, of course, for many points, e. g. behaviour, movement, living observations are invaluable, it cannot be pretended that such can be relied upon where comparative questions of size, form and minute structure are concerned, especially in the case of intra-cellular blood-parasites, which, as is well known, frequently alter or else become deformed in drawn blood.

The next account of Hæmogregarines from lizards was that of Labbé (**13**), who described parasites of this nature from L. muralis, viridis and ocellata. Labbé considered that the various forms which he observed belonged to two distinct genera, to which he gave the names Karyolysus and Danilevskya respectively. With the series of forms comprised in the latter genus we are not here concerned; it is very doubtful whether any are included which should really be kept separate from the ordinary genus Hæmogregarina.[1] In the genus Karyolysus

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[1] It may be noted, however, that Labbé seems to have paid no regard at all to the laws and standards of nomenclature, for he deliberately placed in this genus the parasite of Cistudo europæa, originally described by Danilewsky under the name Hæmogregarina stepanovi, that is to say, the type species of the genus Hæmogregarina; in other words, at his own pleasure, he replaced the generic name Hæmogregarina by that of Danilevskya. If he wished thus to commemorate the Russian savant's name he ought, of course, to have called the parasite which he distinguished as Karyolysus by his name instead. Moreover, for the species of "Danilevskya" which he found in lizards he created the name lacazei, although saying at the time that this was probably the same form as that distinguished by Danilewsky as Hæmocytozoon clavatum. In any case, therefore, this Hæmogregarine of lizards should bear the specific name clavatum (not lacazei), and if it does not belong to the genus Hæmogregarina, the generic name Hæmocytozoon, not Danilevskya, must be given to it.

Labbé placed forms which he regarded as similar to those described first by Danilewsky under the designation H. lacertæ. Why, in so doing, he altered the specific name to lacertarum it is difficult to understand; the name should read, of course, K. lacertæ (Danil.). From a study of Labbé's description I do not think there is any reason to doubt that this author was dealing, in the main, with Danilewsky's parasite, H. lacertæ; though it is true that certain of his figures may represent some other Hæmogregarine. Unfortunately, Labbé does not give any details about the particular species of lizard in which the various types of the parasite he figures respectively occurred. Since he examined four different species of host, in certain of which, at any rate, another Hæmogregarine is also parasitic (as, indeed, he recognised, distinguishing this latter by the name "Danilevskya" lacazei, see footnote, p. 186), it is quite possible that he did not altogether succeed in separating the two forms. Nevertheless, leaving out of consideration his description of the "endoglobular sporulation,"[1] Labbé's account of the appearance, size and structure of the young and adult parasites in the blood-corpuscles, and in particular his description of the marked alterations in the host-cell, make it perfectly evident that most of his observations did actually refer to the same parasite as that described by Danilewsky, and as that which I found in the lizards I examined.

In 1901, Marceau gave an account (**18**) of the Hæmogregarine parasites which he observed in L. muralis, and in this lizard alone; and here also it is quite obvious that the author was dealing chiefly, if not entirely, with K. lacertæ. On the whole, Marceau's description agrees closely with that of Labbé.

It is sufficiently clear, I think, that there is a definite parasite, occurring in L. muralis and probably also in L. agilis and viridis, for which the specific name lacertæ must be retained. Further, in my opinion, it is also preferable to retain Labbé's distinct generic name Karyolysus for this Hæmogregarine, as also for any other similar form which may produce the same characteristic effects upon the host-cell; I certainly consider such forms can be advantageously grouped together—if not in a separate genus, at any rate in a distinct sub-genus—on account of their peculiar behaviour in this respect. It is only necessary to compare the effect on its host-cell produced by an ordinary Hæmogregarina to realise that there is a marked difference between the two types of parasite. Species of the genus Hæmogregarina, whether from fishes or repitles, may often cause more or less hypertrophy of the red blood-corpuscle; but they never stimulate, as it were, the cell-nucleus to undergo such profound changes as is the case with Karyolysus, where the nuclear alteration begins, as I have shown above, almost as soon as the parasite has invaded the corpuscle. I need only refer, by way of illustration, to the recent figures published by Minchin and myself (loc. cit.) of H. triglæ, by Neumann (**23**) of various piscine Hæmogregarines, by Reichenow (loc. cit.) and also Hahn (**9**) of H. stepanovi, and lastly, the figures of many species from snakes given by Sambon and Seligmann (**29**)[1]. In all these cases the host-cell nucleus is practically unaltered; it may be now and then slightly flattened in appearance, but this is usually where it has been pushed to one side of the cell by the growing parasite, and is obviously due to a mechanical cause. It may be said, of course, that if a separate genus Karyolysus is to be thus recognised, the distinction between it and Hæmogregarina will be based mainly, if not entirely, on biological grounds. This is, no doubt, true; but one has not to look far for other instances where a generic distinction. which is generally accepted, is recognised for biological

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[1] This process doubtless represents the schizogony of the parasite, which is apparently either of a double character, similar to that described by Reichenow (**27**) in the case of H. stepanovi, or else of a type where sexual differentiation is already manifest.

[1] Some of these last should clearly be placed in the genus Karyolysus.

reasons, which, if not just the same, are of a similar order. Thus the avian blood-parasite known as Leucocytozoon is distinguished from that known as Halteridium, although there is little doubt that the two types are very similar in structure and in regard to the essential features of the life-history; the principle difference is that of habitat, the one form (Halteridium) being parasitic in the red corpuscles, the other (Leucocytozoon) in the uninuclear leucocytes.[1] Nevertheless, it is very useful to continue to distinguish the two types as separate genera. And similarly as regards these reptilian blood-parasites, as a means of indicating at once the characteristic difference in the effects on the host-cell, it is most convenient to retain the generic names Karyolysus and Hæmogregarina for the karyolysing and non-karyolysing group of species respectively.

Of late years several workers have given accounts of Hæmogregarines from lizards, for the most part recording the occurrence of new parasites—or at any rate, parasites regarded as new—in various additional hosts; several of these are undoubtedly Karyolysus-forms. The parasites of the different species of Lacerta have been studied chiefly by Laveran and Pettit and by França. In their first paper, Laveran and Pettit (**14**) describe the parasites they observed in L. muralis and viridis, more frequently in the former species. They distinguish three different types, all of which they consider to represent Danilewsky's parasite, which they term H. lacertæ; the authors thus use the correct specific name, but prefer to keep the parasite in the genus Hæmogregarina. The first two types are the same as those which I have again found, that is to say, young schizonts and older ones. The only point which requires notice is that the authors consider there is no capsule, but merely a shrinkage space around the second type of form; this is certainly a mistaken view on their part. The third form of parasite is, in my opinion, a type rather different from any phase so far described by other workers, and from anything I have observed. It is a large, curved form, certainly a Karyolysus, because of its effect on the cell-nucleus; I should say it probably represents another phase of K. lacertæ, but until the life-cycle is better known or until this form has been connected by intermediate stages with other known phases, the matter must remain uncertain.

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[1] The different habitat explains, of course, the fact that the one parasite (Halteridium) produces melanin pigment, while the other Leucocytozoon) does not.

França, in a series of papers on the Hæmogregarines of lizards, chiefly species of Lacerta, has been unfortunately preoccupied with the idea that almost every variety in form and appearance of parasite observed represents a distinct and independent type, with the result that he has greatly complicated and confused the subject of these Hæmogregarines of lizards. Thus, in more than one case, the author creates several new species for parasites from the same host, in some instances basing the distinctions between them on such slender grounds as the different staining appearances (tint of colour, presence or absence of granulations, etc.) exhibited. Now, França's figures are all from preparations stained by some modification of the Romanowsky method; and, as is well known, the great variability and uncertainness in the staining appearance presented often by the same object at different times, even where the smear has been treated, so far as was known, in exactly the same manner, renders it perfectly useless to label as distinct species forms showing differences in appearance after being stained by a Romanowsky method, mainly or solely on this ground. Again, França is of the opinion that it is unlikely that a particular species of host will be infected with the same species of parasite in different countries, or even in different districts of the same country. I can only say I do not share this view at all. We know, for example, that Trypanosoma lewisi occurs in rats all over the world; and other common parasites, e.g. certain Gregarines and Coccidia, are known from the same species of host in various countries. I do not think there is any reason to doubt that the same species of Hæmogregarine may occur in the L.

muralis of Portugal, for example, which is found in that lizard in Russia, and again in Southern Austria, and in France. I associate myself entirely with the remarks of Laveran and Pettit in their later note (**15**) with regard to this matter.

In one of his memoirs (**8**), França describes the different forms of hæmogregarine which he found in L. muralis in Portugal. The author leaves out of account altogether the species K. (H.) lacertæ. This he does for two reasons: firstly, in accordance with the view just referred to, because of the different geographical locality of the host in the case of the lizards which he examined; and secondly on the ground that several different forms have been really included in the specific designation lacertæ. From what I have shown above, it will be evident that, on the contrary, we can recognise and clearly distinguish a well-defined species, to which the name lacertæ belongs by right.

França creates no fewer than four new species, all from this one host, namely, H. nobrei, bicapsulata, marceaui and nana. These different parasites usually occur associated together in various groupings; and it is the exception rather than the rule to find them separately. The first three are typical karyolysing forms, and hence may be termed Karyolysus. The last named, it should be pointed out, is, as its name implies, a very small form. From the only figure given it is obvious that this is merely a young phase; it cannot itself be regarded as an adult parasite, and in its older phases it may possibly be identical with one of the other types described. At any rate, it seems distinctly premature, in the circumstances, to give this type a new specific name. As regards França's other three species, I confess straightway that I consider they are only different forms or phases of our old friend K. lacertæ. I have come to this conclusion principally on two grounds; in the first place as a result of the detailed comparison I have myself made of certain forms of K. lacertæ, and of the alterations produced in the infected host-cells as seen in smears stained with Giemsa and also in wet preparations stained with iron-hæmatoxylin; and secondly, as a result of the valuable light thrown on the whole subject of the life-cycle of a Hæmogregarine by Reichenow's work on H. stepanovi. Of course, this work has appeared since França's papers were published, so that we have now a guide to the interpretation of the various phases which was then unavailable. As the general scheme of the life-cycle, so far as it is undergone in the Vertebrate host, has been shown by Miss Robertson (**28**) to be fundamentally similar in the case of another Hæmogregarine also, I think we may regard it as probable that the life-cycle is similar, in its main traits, in other reptilian Hæmogregarines; and there is no need to consider that of Karyolysus as likely to be very different from that of Hæmogregarina merely because of the biological differences between the two forms, i.e. with respect to the behaviour and reaction of the host-cells. Assuming a general agreement, a particular type or stage of parasite observed in a lizard might represent any of the following phases in the life-cycle of a single species: The young growing schizonts produced from the sporozoites in a new infection; the merozoites or growing schizonts resulting from a first type of schizogony, e.g. with many merozoites (micromerozoites?); the merozoites or young schizonts resulting from a second type of schizogony, e.g. with few merozoites (macromerozoites?); lastly, the growing gametocytes, which may themselves be differentiated. As these various phases very likely show definite, though it may be slight distinctions from one another, if they were only observed casually, as it were, and their further development was not followed, nor their connection with one another ascertained, some would at once jump to the erroneous conclusion that they constituted distinct and new species.

Considering França's three Karyolysus-forms separately, K. (H.) bicapsulata, which we may take first, is so named because of two caps of deeply staining matter which occur one at each end of the parasite. From França's fig. 7 it is seen very clearly, in the first place, that these "caps" are distinctly outside the true envelope or capsule of the Hæmogregarine, and secondly, that they resemble closely in appearance the hypertrophied nucleus of the host-cell, and, in fact, may be connected with the latter (for instance, the cap on the right-hand side of the upper parasite of fig. 7). Now, as stated above, I have observed a very similar appearance in some individuals of K. lacertæ in Giemsa-stained smears (cf. figs. 16–18). In my opinion there is no doubt whatever that these "caps," in the case of França's parasite also, are simply the result of the alteration to the nucleus, the thicker or club-shaped end-parts of which curve round the parasite and may be almost or quite detached from the middle portion; these caps have nothing whatever to do, directly, with the parasite. A perfectly similar behaviour of the nucleus of the blood-corpuscle has been described by Billet [**2**] in the case of K. (H.) curvirostris; two of this author's figures show exactly the same condition. Other points about França's account of K. "bicapsulata," e.g. the average size, the presence of a definite envelope around the parasite, make me practically certain in my own mind that this is not a new species at all, but only a phase of K. lacertæ corresponding to the second, older type described above. I should add, however, that Laveran and Pettit also seem to regard this "bicapsulata" as a distinct species, although they say that they found it associated with lacertæ, and mention further that, in deeply stained specimens, the "caps" stain very similarly to the deformed host-cell nucleus!

K. (H.) nobrei. This form Laveran and Pettit (loc. cit.) themselves consider resembles K. lacertæ so closely that it is doubtful whether it is really a distinct species. In my own preparations I have not come across any individuals which exactly represent this form; the parasite drawn in fig. 15, however, shows considerable resemblance in size and general appearance to the form figured by França in his fig. 2, the chief difference being in the position of the nucleus, which is near the middle of the parasite in França's case. I should say it is very likely that this is just one of those cases referred to above, where a different phase in the life-cycle of the parasite has come under observation. From a consideration of França's figures relating to K. nobrei, the suggestion may perhaps be put forward that the phase in question corresponds to the second process of schizogony (with few merozoites) which occurs in H. stepanovi, and the type of individual immediately preceding or resulting from the same.

Again, with regard to K. marceani, a form occurring in the blood is practically indistinguishable, according to certain of França's figures, from some individuals of the second type of K. lacertæ, which I have described; thus my figs. 9 and 12 agree closely with his figs. 9 and 10 respectively of K. marceaui. França also mentions and figures certain phases from the liver, which he considers represent conjugation. What these do exactly signify is uncertain, but the micro- and macrosporogony described as resulting from this process is quite comparable to Marceau's account of the same process in what is admittedly K. lacertæ. (It may be added that in both cases it is of course much more probable, considering the matter in the light of Reichenow's work, that schizogonic multiplication is concerned.) Hence, on the whole, and at any rate until the life-cycle of K. lacertæ has been thoroughly worked out, it is very much better, I think, not to adopt these new names, bicapsulata, nobrei and marceaui, which would only entail great confusion and difficulty, but to consider them as representing merely different phases of K. lacertæ.

To complete my summary of this question, I must mention that there has been the same prematnre and probably useless multiplication of species in the case of Karyolysus-forms from another species of Lacerta, viz. L. ocellata. In the first place, Billet [2] gave a short account, already referred to, of a karyolysing Hæmogregarine occurring in this lizard in Algeria, to which he gave the specific name curvirostris. As this parasite occurs in a different species of host, we may perhaps assume for the present that it is a form distinct from K. lacertæ, though I do not think this can be regarded as at all certain. A few weeks later, Nicolle [24] also described a similar Hæmogregarine, from a variety of L. ocellata in Tunis, which he considered to be distinct from curvirostris and called biretorta. Lastly, França [7], not content with these two, makes three additional species, H. [K.] schaudinni, nicollei and minuta, to say nothing about a variety africana of his first one, all from L. ocellata. Thus in two species of Lacerta, namely muralis and ocellata, there are according to França no less than ten species of Hæmogregarine. Is not this carrying species-splitting to an absurd degree?

I have not studied the parasites of L. ocellata myself, but having regard to the above analysis of the so-called species of L. muralis, some of those from this other lizard must be viewed with great suspicion. For instance, biretorta is almost certainly the same parasite as curvirostris, and hence a synonym of the latter; this is clear to my mind, from França's figs. 15 and 17 (loc. cit.), and, indeed, Laveran and Pettit, in a note I have not been able to see,[1] have also thrown doubt upon the independent nature of biretorta. The same conclusion applies to França's species nicollei, which the author himself admits has considerable resemblance to curvirostris and biretorta; in short from França's fig. 18 it is obviously only a slightly different phase of K. curvirostris. The parasite termed by França schaudinni appears rather different in character both from lacertæ and curvirostris, although França's fig. 2 of this form is remarkably like my fig. 4 of K. lacertæ; it may perhaps be left an open question whether schaudinni is some other phase in the developmental cycle of K. curvirostris or a distinct species. It is rather odd, however, that França has included as a particular form of curvirostris a type (vide his fig. 16) which is undoubtedly only a form of his schaudinni! I conclude the subject by registering a strong protest against this habit of creating a new species on entirely insufficient grounds.

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[1] 'Bull. Soc. Path. exot.,' ii, 1909, p. 377.

III. COMPARISON OF THE NUCLEAR CONDITION IN KARYOLYSIS LACERTÆ AND CERTAIN OTHER HÆMOGREGARINES WITH THAT OF COCCIDIA; THE QUESTION OF THE KARYOSOME AND THE INTRA-NUCLEAR DIVISION-CENTRE.

I propose next to compare the nuclear condition, as I have described it above in Karyolysus lacertæ, with that which is found in certain Coccidia, at a particular period in the life-cycle, since, in my opinion, the agreement shown affords an important indication of the close affinity and phylogenetic relationship of these two types of parasite. This resemblance is especially marked in the case of the merozoites and very young schizonts of a Coccidian, which is, according to Shellack and Reichenow (**32**) really Barrouxia alpina, Léger; this phase, it must be mentioned, has for long been mistakenly included in the life-cycle of Adelea ovata, of which it was considered to represent the male type of schizogony. The structural details of this particular stage or form of the parasite were first described by Siedlecki (**33**), and further notes with regard to it have since been given by, among others, Jollos (**12**), both these authors having included it in the cycle of Adelea.[1] In order to facilitate the comparison with the Hæmogregarine, I have drawn (Pl. 9, figs. 41–43; Pl. 10, figs. 1–3) some individual merozoites from an original preparation of my own, these parasites being easily obtainable in centipedes. Although I have found exactly the same nuclear condition and behaviour in this early phase which has been observed by Jollos, I think it is worth while to describe it again, because doubts have been recently cast upon Jollos' account, both as regards these points and others.

At first the young schizont of Barrouxia, which may be little more than a merozoite,[1] has a single large karyosome placed quite at one side of the general nuclear substance; the latter is finely granular in character, and does not stain deeply, the granules being fairly uniform in size and appearance. More frequently a rather later condition or phase is found, in which there are two karyosomes, generally at opposite sides of the principal nuclear mass; these two karyosomes are usually more or less unequal in size, and neither is so large as when there is only one. I have been much exercised in regard to the question of the true situation of these karyosomes. In nearly all the individuals a well-marked clear zone, which in some cases is relatively wide, surrounds both the general nuclear substance and the karyosomes (or karyosome). Is this clear zone to be considered merely as a shrinking-space, separating the whole of the nuclear organellæ from the general cytoplasm of the parasite, or is this area really within, and therefore a part of, the nucleus, the limit or border of which is on the outer side of the clear area and in contact with the edge or margin of the surrounding cytoplasm? In the former case, of course, the karyosomes would be actually extra-nuclear; in the latter they would be within the nucleus, but excentrically placed, near the periphery. After some hesitation I have come to the conclusion that the latter view is the correct one, and that the pale, clear area really constitutes the peripheral region of the nucleus. In the case of most individuals I have found it almost impossible to satisfy myself of the existence of a definite membrane, bordering this zone externally, as distinct from the edge or margin of the surrounding cytoplasm itself; and the same difficulty has presented itself apparently to other observers, if one may judge from certain of their figures (e.g. Siedlecki's fig. 17 and Jollos' figs. 22 and 28). Moreover, the limit of the centrally situated, uniformly granular, nuclear material is at times so sharp and well-defined that it might almost be regarded as a membrane. However, now and again one is fortunate enough to be able to focus a definite line bordering the pale area in question on the outside, which most probably represents a true nuclear membrane. And there are one or two other reasons which support this view. Thus Siedlecki (loc. cit.) states that he observed this clear zone in these forms of the parasite even in the living condition, which shows that, in the strained preparations, it cannot represent merely a shrinkage-space. Further, although this zone appears so clear and pale by comparison with the parts of the parasite immediately surrounding, it is, nevertheless, occupied by something—probably in the nature of nuclear sap—which is extremely faintly stained; that this is actually the case is sometimes shown distinctly because of a peculiar condition or appearance which is often, but not always, presented by the karyosomes. These elements themselves, especially the larger ones, i.e. when there are only one or two, may be surrounded by a perfectly clear halo-like circle, which is quite colourless; this halo round the karyosome passes between it and the central nuclear substance, indenting the surface of the latter, so that it forms a concavity or cup as it were. The difference between this small, quite colourless zone and the almost clear, faintly staining area, extending around the periphery of the whole nucleus is sufficiently conspicuous. To sum up the matter, therefore, the karyosomes must be considered as really intra-nuclear, situated in a peripheral zone, which is very pale, and apparently consists only of nuclear sap, the rest of the nuclear material, containing a small amount of chromatin being aggregated to form a central mass. I have not been able to see any delicate threads or rays passing from this central mass to the limiting membrane of the nucleus, and traversing the faintly-stained, peripheral zone, nor does Jollos (loc. cit.) mention or figure anything of the kind; but Chagas (**5**) has described and figured "linin threads," having such a disposition in the case of somewhat older phases of a new species of

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[1] The two forms are parasitic in the same host, Lithobius forficatus; this fact is, of course, chiefly responsible for their different phases having been confused together.

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[1] The earliest change in the condition of the karyosome, namely its division into two, may even take place before the fully formed merozoite has been liberated from the "barillet" of which it has constituted a segment.

Coccidian ("Adelea" hartmanni),[1] in which the nuclear constitution and behaviour of the young schizont is very similar (cf. also below, Note IV, where the nucleus of Leucocytozoon is compared).

To return now to the behaviour of the karyosome. The two subequal or unequal karyosomes result undoubtedly from the division of the original large, single karyosome, which takes place in a promitotic manner; for in a couple of instances I have found the centrodesmose still persisting (cf. fig. 42). There is no possible doubt about this division of the karyosome; the process here appears to be just the same as in Karyolysus lacertæ, and my having found it in both parasites substantiates and corroborates Jollos' account of this behaviour of the karyosome in the young schizonts of this Coccidian. While the early condition and behaviour of the karyosome during this period is thus completely paralleled by the above-described early phase of K. lacertæ, the subsequent course of events differs slightly in the two parasites. In the Coccidian, at a rather late stage, three or four karyosomes are present (fig. 43, also fig. 3, Pl. 10), most of which are small and have obviously arisen by the further division of one or both of the two above-mentioned daughter-karyosomes (cf. also Jollos' figure).[2] That is to say, here the karyosome continues to be separate and distinct from the general nuclear substance (as is known from the ascertained further development), whereas in K. lacertæ the karyosomatic chromatin which is retained by the nucleus becomes distributed amongst the general chromatic substance and no longer distinguishable.

It is necessary to emphasise this fact of the promitotic division of the karyosome because, in recent papers, Reichenow

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[1] This parasite is regarded by Léger (**16**) as the type of a new genus, Chagasia.

[2] It may be recalled that Siedlecki himself, in his original description of this form, also states that the karyosome divides: thus, "il [le karyosome] donne, par bourgeonnement, naissance à des karyosomes secondaires," and, again, "surtout un karyosome, parfois divisé en deux ou trois fragments."

(**27**) and Schellack and Reichenow (**32**) have maintained that no division of the karyosome occurs in the above phase of Barrouxia ("Adelea"), and consider that the secondary karyosomes (i. e. the daughter-karyosomes) arise de novo, by independent formation from the general nuclear substance ; in regard to this detail the authors are certainly mistaken. Moreover, quite recently Chagas (loc. cit.) describes and figures, in his account of Chagasia (Adelea) hartmanni, perfectly similar promitotic divisions of the karyosome in different phases of the life-history. I have a strong idea that Reichenow and Schellack, in arriving at the above conclusion, have been influenced—if unconsciously—by the view which one of them (Reichenow) seems to have formed upon the question of the karyosome, its nature and significance, as a result of his work on Hæmogregarina stepanovi. No one is more sensible than am I of the great value of Reichenow's research, which has thrown full light upon the complicated subject of the Hæmogregarine life-cycle ; but in regard to this somewhat important cytological question I find myself obliged to differ from him.

Hartmann and Chagas (**10**) have suggested that the reason for this may be that as the particular parasite (Hæmogregarina stepanovi) upon which Reichenow worked is a very small one, the observation of minute cytological details and changes would be rendered more difficult and hence they may have escaped detection. I do not altogether share this opinion ; for one thing, I do not think H. stepanovi is much, if any, smaller than the small forms of K. lacertæ, where the karyosome and its division can be made out without difficulty. I am inclined to consider that, on the whole, the nuclear constitution and behaviour in H. stepanovi is as Reichenow has described it ; and therefore, as a logical sequel, that this species of Hæmogregarine differs in one or two cytological respects, such as the absence of a typical karyosome, from certain other Hæmogregarines and certain Coccidia. This is the more probable, in my opinion, because of a fact which is evident on scrutinising Reichenow's figures, namely, that the chromatin of the general nuclear substance is very much more prominent, i.e. in the form of numerous fairly large, deeply staining grains, than is often the case in the corresponding phases of other parasites where a karyosome is present; and just the same condition is seen in the closely allied species, H. nicoriæ, according to Miss Robertson's description (loc. cit.). If the nuclear appearance of these parasites is compared with that, for instance, of the young phases of either K. lacertæ, H. gracilis (Wenyon [**36**]), H. lutzi (Hartmann and Chagas [**10**]), or of Barrouxia alpina ("Adelea ovata") or Chagasia hartmanni, a striking difference is at once apparent; in the latter, most, sometimes nearly all, of the chromatin is contained, for the time being, in a distinct karyosome (or more than one). It is especially in regard to this absence of a definite karyosome that the two species of Hæmogregarine from tortoises are interesting. Thus, Miss Robertson expressly states that "at no stage does H. nicoriæ show in its nucleus the karyosome so characteristic of Coccidia." Now, in my opinion, H. stepanovi shows an important intermediate condition between the type of nucleus possessing a karyosome, as in the above examples, and a type like that of H. nicoriæ, where this organella is quite wanting. According to Reichenow, H. stepanovi has at certain periods of its life-cycle (which, in general, correspond to the phases when a karyosome is present in other forms) a definite rounded body, situated near the periphery of the nucleus, which is always very pale and faintly stained and appears quite different from the prominent chromatic grains.

Reichenow uses the term "nucleolus" for this body, and this is most probably the correct name for this particular structure, and indicates its true nature; but my reason for thinking so is not exactly the same as that given by Reichenow. It seems clear from the author's description and figures that the body in question contains little or no chromatin; it corresponds apparently to the true nucleolus of an ordinary tissue-cell, i.e. a body consisting simply of plastin or allied material. Reichenow, however, regards this element as a nucleolus principally on the ground of its behaviour during nuclear division, that is to say, its disappearance and re-formation at different periods. Unfortunately, Reichenow's observations on this body in H. stepanovi, which have led him to the conclusion that it has the physiological significance of an ordinary nucleolus, have prejudiced his view upon the true karyosome, which is something quite different. He has, in my opinion, failed to grasp what is the really essential feature of a true karyosome, namely, that it is a chromatin-nucleolus, an organella which holds or contains a large proportion of the entire chromatic substance of the nucleus. His only reference to this fundamentally important character is seen in the following sentence:—"Was ihn [d. h. den Binnenkörper (Karyosom)] von dem echten Nucleolus unterscheidet ist, abgesehen von seinem Chromatingehalt auf den wir keinen grossen Wert legen dürfen, allein der Umstand, dass er bei der Kerntheilung erhalten bleibt" (the spacing is mine). Because he thus ascribes no importance to this, the principal feature of the karyosome, he is able to persuade himself that the typical "Binnenkörper" or karyosome in other cases is the equivalent, practically speaking, of the body he has described in H. stepanovi.

Further, Reichenow brushes aside as quite untenable the usually accepted view that the karyosome behaves as an intra-nuclear division-centre, which is founded on the reliable observations of many previous workers. The admitted existence of the "Hantel-Figur" he endeavours to explain by supposing that it is produced by the karyosome being drawn out into two parts by the separating halves of the dividing nucleus. He appears to have adopted this attitude on two grounds: in the first place, because he has found that the nucleolus of H. stepanovi does not so divide, and secondly, because he evidently doubts the existence at all of an intra-karyosomatic centrosome and the occurrence of promitotic division, so far as the Coccidia and Hæmosporidia are concerned. In regard to the first point, the very fact that the organella seen in H. stepanovi is a nucleolus and not a karyosome explains why it does not divide, as I hope to show below (cf. pp. 213 and 214).

With regard to Reichenow's doubts about the occurrence of promitotic division and the presence of an intra-karyosomatic centrosome, I must say I think they are quite unfounded. In the first place, both my own observations on the same Coccidian and those of Chagas on an allied form support Jollos' account (loc. cit.) in so far as regards this detail. Further, I have found a precisely similar division of the karyosome by means of a centrodesmose in an early phase of the Hæmogregarine, Karyolysus lacertæ.[1] And, as I have previously remarked, the presence of a centriole within the karyosome may be legitimately and reasonably assumed where the occurrence of a centrodesmose is noted. From a study of Trypanosomes, I know how difficult it often is to actually distinguish the centrosome, even in the large karyosome of a relatively large individual, although the occurrence of a centrodesmose in the division of the karyosome (e. g. of the trophonucleus) has long been well known. Nevertheless, Minchin and I, in our notes on T. raiæ (**20**), clearly demonstrated the actual presence of a centrosome in the resting karyosome. Moreover, as regards the Hæmogregarines, since Reichenow's paper appeared, some interesting observations on the leucocytic parasite of the dog, Hepatozoon (Hæmogregarina) canis, have been published by Wenyon (**37**). Here, too, a distinct promitotic division of the karyosome is figured; and in the case of this parasite, the karyosome is relatively very small in some phases, when it probably represents little more than the centrosome itself. Even in the nucleus of H. stepanovi, it is not impossible that a centriole is really also present, and it is just in regard to this detail that I think the suggestion of Hartmann and Chagas (**10**) may apply, namely, that this minute granule may have escaped recognition owing to the difficulty of distinguishing it amid the more prominent chromatic grains. In this connection it must be noted that Miss Robertson (**28**) mentions and frequently figures a small but definite granule in the nucleus of H. nicoriæ, which is in no way distinguishable from the peripheral chromatin grains in size or staining reaction, but which nevertheless appears to be different from the other nuclear elements in so far that, in the primitive type of nuclear division, it seems to form a centrodesmose. This minute body may well be the centrosome; just as the central granule which I have sometimes noted in the nucleus of K. lacertæ, when there is no longer a distinct karyosome, is also probably one (cf. fig. 40).

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[1] Cf. also footnote to p. 205.

It is a pity that Reichenow, in his able memoir, should have thought himself at liberty to disregard or treat as negligible the evidence afforded by the research of other earlier workers, such as the classic instances of Coccidium schubergi and Cyclospora caryolytica, made known by Schaudinn (**30** and **31**), which pointed clearly to the existence of this characteristic promitotic division of the karyosome in the respective parasites, and which has since been abundantly corroborated in other cases; to say nothing of his having entirely failed to take into consideration that in several of the lower Flagellates the occurrence of a centrosome and of promitotic division of the karyosome is now well established. As it is generally agreed to-day that the Ectospora (Telosporidia) are descended from Flagellate ancestors, it might be expected, on à priori grounds alone, that among Coccidia and Hæmosporidia some would be found to exhibit a similar mechanism in their nuclear division.

I certainly do not think it is advisable to adopt such a comprehensive generalisation as that postulated by Hartmann and Chagas and the followers of their school, namely, that a central organella (centrosome) is present, as a general rule, in the karyosome of all Protozoa; but I will at once admit that I consider this idea considerably nearer the truth than the view maintained by Richenow, that a centrosome is not present in the karyosome in any of the cases mentioned above, and that no promitotic division of this body occurs.[1]

Nature and Significance of the Karyosome.

I have laid stress upon this fact of the presence of an intrakaryosomatic centrosome because of the important bearing it has upon the question of the real nature and significance of the karyosome, and because it helps to explain satisfactorily the different behaviour of this body in different phases of the life-history. In the first place, it is necessary to clear the ground of what I consider is a serious misconception of the karyosome, which is largely fostered by the school of Hartmann, Nägler and others, and which appears to be based upon the fact that this organella frequently leads the way in nuclear division, and contains within itself a division-centre. Now, the primary and principal meaning of the term karyosome is chromatin-nucleolus, i. e. a body consisting of a plastin basis impregnated with chromatin; it might be considered unnecessary at the present day to have to emphasise this essential character, but that this is not so is shown by Reichenow's reference to it as one "auf den wir keinen grossen Wert legen dürfen!" This is the sense in which the word was first used, and on account of which it has been adopted by most authors (cf. Labbé (**13**), Minchin (**19**), Siedlecki (**33** and **34**), Wilson (**38**) and others). Schaudinn, in his celebrated memoir on the Coccidia of Lithobius also says: "Jeden Falls unterscheidet sich das Karyosom der Coccidien von den echten Nucleolen der Metazoenzellen scharf durch seinen Chromatingehalt." But in many recent papers by members of the school of thought referred to above, a strong tendency is noticeable to assume that the possession of a centrosome and of the function of acting as a division-centre is to be definitely associated with the idea of a karyosome as a whole and to be implied in the meaning of the term, as a definite attribute of this body; thus, Hartmann and Chagas (**11**) say: "Man kann daraufhin jetzt den Begriff des Karyosoms direkt von dem Vorhandensein eines Centrioles [Centrosoms] abhängig machen." This notion has been elaborated to such an extent that the whole karyosome, that is to say, chromatin-nucleolus + centrosome, has come to be regarded as a distinct entity, a locomotor or kinetic centre; its chromatin is the "kinetic component," while the surrounding chromatin, scattered through the nucleoplasm or nuclear sap, is the "generative component" (the second nuclear type of Hartmann and Chagas).

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[1] See also the account given in Note IV of the nuclear structure of Leucocytozoon and Halteridium, in both of which division-centre and centrodesmose are clearly shown.

Now, in my opinion, this idea of the karyosome is very forced, besides being really quite unsupported by any evidence. For one thing, I do not consider that the whole karyosome (i.e. chromatin-nucleolus + centrosome) can be regarded as representing a definite unit or "locomotor-centre"; it may happen, in fact, that the intrinsic division-centre is outside and distinct from the karyosome (as in Spongomonas, for example, figured by Hartmann and Chagas, and cf. also the "nucleo-centrosome" of Adelea zonula, according to Moroff (**21**). Again, the condition shown by the true Binucleata, the Trypanosomes and their allies is quite against this interpretation. Here there are two separate nuclei—a locomotor nucleus (kinetonucleus) and a vegetative one (trophonucleus); to this, of course, Hartmann and Chagas assent, saying (loc. cit.) that "zwei verschieden differenzierte Kerne in der Zelle vorhanden sind, einer [trophonucleus] vorwiegend mit der trophisch-generativen Komponente, der andere [kinetonucleus] vorwiegend mit der lokomotorischen Komponente." But nothing is more certain than that the trophonucleus of a Trypanosome possesses a large, conspicuous karyosome, containing most of the chromatin of the nucleus, and also a distinct centrosome (centriole)! If, therefore, the karyosome in this case is a trophic component (which is, indeed, the most reasonable view to take), whatever ground is there for supposing that, in the passive, intra-cellular Coccidian, the equally large and conspicuous karyosome represents a kinetic (locomotor) component? Moreover, another idea prevalent in the writings of the adherents of this school, which is strongly to be deprecated, is that of contrasting, as two opposed constituents, kinetic and generative components of the nucleus. These two things are not strictly comparable or opposable at all. On the one hand, the essential kinetic components are the achromatic elements —centrosome, centrodesmose, and so on; and in all probability these take part in effecting the division of generative chromatin as well as of vegetative (trophic) chromatin. And, on the other hand, where a separate kinetonucleus is present, which may be regarded as standing in a special relation to the locomotor activities of the Trypanosome, there is no reason whatever for supposing that the chromatin of this nucleus is less generative in character than that of the trophonucleus.[1] In short, I cannot share the above view of the locomotor or kinetic nature of the karyosome as a whole at all; it is the contained centrosome, not the chromatin-nucleolus, that brings about the division. The so-called "Hantel-Figur" is really the result of the gradual (passive) separation of the two halves of the karyosome as the centrodesmose extends.[2]

It seems to me very much better to return to the earlier manner of regarding the karyosome, which has been well set forth and discussed by Siedlecki (**34** and **35**), namely, that it is an organella, whose principal function is to store up reserve chromatin—and particularly trophic as distinct from generative chromatin—for use as and when required by the nucleus, or, as the case may be, for elimination if not required. This theory undoubtedly fits in best with the known variations in behaviour of the karyosome at different periods of the life-cycle. For instance, as regards the Coccidia, speaking generally it may be said that during the schizogonic, vegetative phases, the karyosomatic chromatin becomes subdivided up, in a promitotic manner, amongst the daughter-individuals; on the other hand, as a rule, on the approach of the sporogonic part of the cycle—frequently during gametogony or else early in the history of the zygote—the karyosome is mostly eliminated, a "nuclear purification" of the unrequired, trophic chromatic material taking place.[1] Moreover, in connection with this view, a very simple explanation can be offered of the presence of an intra-karyosomatic centrosome, one which appears to me to render quite unnecessary the involved conception of the karyosome discussed above. It must be remembered that the promitotic type of division, which is the type found where the centrosome is contained within the karyosome, is of a primitive character, as its name implies. It is most likely that the reason why the centrosome, i.e. the intra-nuclear division-centre, is inside the karyosome in such cases is simply because the latter body does contain, for the time being, the larger proportion, or it may be nearly all of the chromatin of the nucleus, the division of which it is the function of the centrosome to bring about and regulate; in other words, because, having regard to the primitive character of the mechanism, the function of the division-centre is the better performed the more intimately it is associated with the chief chromatin-containing constituent of the nucleus.

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[1] This point was emphasised by me so long ago as 1906 in my analysis (**40**) of Schaudinn's celebrated work on the parasites of the little owl.

[2] The same interpretation is in all probability to be applied to the "nucleolo-centrosome" (Keuten) of Euglena, especially as Hartmann and Chagas (loc. cit.) have shown that promitotic division of the karyosome, by means of a centrodesmose, occurs in another Englenoid, Peranema trichophorum.

Further, on this view a separation of centrosome and karyosome, as the nuclear development reaches a slightly more advanced stage, would be readily intelligible. Such an occurrence of the division-centre distinct from, or independent of, the karyosome (but at first, of course, remaining intra-nuclear) may have been brought about in more than one way. Thus it may be the result of a more elaborate development of the mechanism of division; an example of this is seen in the case of Spongomonas, to which reference has previously been made, where the centrosome passes out of the karyosome at the period of division and a definite mitotic figure is formed. Or, on the other hand, it may be due to the development of another type of nuclear structure, where, either during certain phases in the life-history or throughout the whole cycle, there is no longer a karyosome present in the nucleus as such, but the chromatin is more or less uniformly distributed on a reticulum throughout the general nuclear substance, in the middle of which the centrosome may persist.[1] And it is in this direction that the nuclear constitution has apparently developed in the Hæmogregarines. Lastly, a further stage in nuclear evolution would be reached by a combination of the two lines of development indicated, i. e. by the elaboration of the nuclear structure itself, associated with a more perfect development of the division-mechanism; and thus a condition might be arrived at such as is seen in the daughter-nuclei formed during the period of nuclear multiplication, which precedes gamete-formation, in many Gregarines (cf. the figures of Brasil [**4**], Léger and Duboscq [loc. cit.], Woodcock [**39**]), where we find perhaps the highest grade of nuclear constitution and mode of division attained among the Sporozoa.

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[1] It may be noted that Léger and Duboscq (**17**), in their admirable account of the sexual processes among Gregarines, also adopt this interpretation of the elimination of karyosomatic material.

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[1] It is important to note that even where a division-centre is certainly present during particular phases of a life-cycle, this may nevertheless be wanting, or at any rate not recognisable, during other periods of the same life-cycle. Thus, in many Coccidia (e.g. Coccidium schubergi, Cyclospora karyolytica, according to Schaudinn), the division of the definitive nucleus of the zygote to form the sporoblast-nuclei is direct; but, on the other hand, in Adelea (cf. A. ovata, mesnili [Perez, **25**] and hartmanni) the sporogonic divisions appear to be promitotic, i.e. more or less comparable to the schizogonic ones, allowance being made for the absence of a karyosome). Again, in the nuclear divisions of the sporont of the Gregarine, Diplodina irregularis, I have shown (**39**) that the first ones are direct (amitotic), the later ones mitotic.

We are now in a position to summarise, comparatively, the various types of nuclear condition which have been described in different Hæmogregarines. In Karyolysus lacertæ a definite karyosome is present in the youngest schizonts. This undergoes promitotic division which is usually unequal. The smaller half divides again, and the resulting portions ultimately become incorporated with the general nuclear material; the larger half of the karyosome, on the other hand, is eliminated from the nucleus and passes to the surface of the body-protoplasm, becoming altered and probably partially used up by the cytoplasm in its passage.[1] As already mentioned, I am of the opinion that the division-centre persists in the modified nucleus and can be seen at times as a definite central granule. I am unable to say whether a karyosome is developed again in a later phase of the life-cycle. In Hepatozoon (Hæmogregarina) canis, according to Wenyon (**37**), the karyosome persists throughout the schizogony, its division occurring in the usual promitotic manner; in this case, the body regarded by Wenyon as a karyosome is very small comparatively, and, I should say, represents little more than the intra-nuclear division-centre itself. Wenyon does not mention whether he observed any elimination of chromatic material before or during schizogony. On the other hand, in Hæmogregarina nicoriæ a karyosome cannot be distinguished at all, the nucleus appearing in all phases to have its chromatin more or less regularly distributed upon a reticular framework; a definite intra-nuclear centrosome is regarded, however, as being present. H. stepanovi shows, as I consider, a very interesting stage in the disappearance of the karyosome as a distinct organella. In certain phases a nucleolus is present, occupying the same excentric or peripheral situation in the nucleus which is occupied in other forms (e.g. Karyolysus, "Adelea") by the karyosome. I suggest that this element represents the plastin basis of an ancestral karyosome, the chromatin which it originally stored having become now (permanently) distributed through the general nuclear material in the form of numerous large conspicuous grains.[1] In this connection an observation made by Reichenow is significant. He found that in the young growing schizont, chromatic substance is regularly eliminated from the nucleus and cast out of the cell-body of the parasite, i.e. a precisely similar occurrence to that seen in Karyolysus and Barrouxia sp. Reichenow is uncertain whether it is the nucleolus ("Binnenkörper") which is thus got rid of; but, as he himself points out, the fact that the nucleolus is always very faintly stained, while the expelled element stains on the contrary deeply and is manifestly chromatic in origin, is against this view. Moreover, I may point out that in slightly older schizonts again, the nucleolus is still present in the nucleus (cf. Reichenow's figs. 73–75). Hence it is more probable that this eliminated chromatic substance is derived from the general nuclear chromatin. As this process here doubtless has the same object as the corresponding one in other parasites, the inference is that the chromatin which in other cases is stored up in the karyosome is in H. stepanovi incorporated with the rest of the chromatic material of the nucleus, the plastin basis of the karyosome alone remaining. On this explanation, and having regard to the views I have expressed above, it is readily understandable why the nucleolus does not divide, with the formation of a "Hantel-Figur," a fact which appears to have puzzled Reichenow. There is no need for a division-centre to be present in the nucleolus because it no longer possesses the chromatin of a karyosome (chromatin-nucleolus). If, as seems to me quite possible, a centrosome does occur in H. stepanovi, this is most likely to be in the centre of the chromatic network of the nucleus.

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[1] It is instructive to note that a similar elimination of karyosomatic material before the young schizont proceeds to nuclear multiplication is described by Averintzeff (**1**) in the case of Barrouxia sp., parasitic in Cerebratulus. The process may apparently take place according to one of two slightly different modes, the second of which furnishes a close parallel to the nuclear behaviour of the corresponding phase in Karyolysus.

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[1] So prominent are these grains and apparently in certain phases usually of a fairly constant number (i.e. within limits) that Reichenow is inclined to regard them as definite units comparable to chromosomes.

Before concluding this section, I should like to add a few remarks about the nuclear condition seen in the piscine Hæmogregarine, H. triglæ, to make a comparison with which was my original intention in commencing to study the nucleus of K. lacertæ. Minchin and Woodcock (loc. cit.) found that in both the small forms and the two large types of the parasite one or two large grains are frequently, though not invariably present, situated either close to the nucleus, or some varying distance from it; these bodies are very deeply stained and prominent in films stained by iron-hæmatoxylin. The nucleus itself appears comparatively pale and consists of small grains of chromatin, often somewhat faintly stained, on an irregular network. In Giemsa-stained smears it is difficult to distinguish this grain (or grains) when close to the nucleus. In our paper describing H. triglæ we regarded these elements as not chromatic, but rather of the nature of centrosomes. The extra-nuclear position of the body, together with the fact of its being often paired, seemed to us very much against its representing a karyosomatic element. Moreover, the appearance of these grains after being stained with Twort's stain did not, in our opinion, furnish sufficient evidence in favour of their being chromatic. It is true that in freshly made preparations they were often stained red, i.e. with the neutral red, the chromatin staining constituent of Twort; but they had no strong affinity for the red, because in preparations which had been made some time the red tint had quite vanished from them, although the nucleus itself retained the red colour. I think we were misled by this behaviour after Twort. While it may be said that only chromatic elements are stained red by this stain, I think now that it is nevertheless quite likely that chromatin in some states or conditions may possess only very slight affinity for the neutral red.

Discussing at the time the question of the Hæmogregarine nucleus, we considered this to be of a distinct type, entirely lacking a karyosome. Börner (**3**), in his account of Reptilian Hæmogregarines (the best, as regards cytological details, which had been published up till then), had expressly stated that he never in any case found a karyosome present. Moreover, mentioning the matter in conversation with Miss Robertson, she also agreed that the Hæmogregarine upon which she was at the time working (H. nicoriæ) also had no karyosome associated with its nucleus. The only mention in the literature up to then of the occurrence of a karyosome in the nucleus of a Hæmogregarine was by Wenyon (**36**), in the case of certain phases of H. gracilis, from the liver of Mabuia. It appeared to us at that time highly probable that Wenyon had mistaken phases of some Coccidian parasite of the liver for phases of the Hæmogregarine, particularly as other, rather similar stages figured by Wenyon, which were undoubtedly referable to the life-cycle of H. gracilis, showed no karyosome in the nucleus. In the light of the observations discussed in the present paper, I willingly admit that our opinion was very probably mistaken, and that Wenyon may have been quite right in attributing all the phases he figured to the life-cycle of H. gracilis.

In short, it is now perfectly clear that the Hæmogregarine nucleus cannot be considered as being of a distinct type, but that, on the contrary, it shows close agreement with, or is easily derivable from, the Coccidian nucleus. Either a definite karyosome is present, at all events during some part of the earlier (schizogonic) phase of the life-history, when it behaves in a manner quite parallel to what is found in certain Coccidia, or else its complete absence is readily accounted for by a consideration of its behaviour as the development proceeds in those parasites in which it does occur.

Therefore, in the case of H. triglæ, it is most probable that the conspicuous grains also represent karyosomatic elements, and that they do contain chromatin in some form or other. In our preparations we did not observe any division-figures, but it is not unlikely that where two grains are present, they have originated by the division of one, if comparison is made with the somewhat similar condition seen in Kalyolysus and "Adelea." Whether, again, a portion of the chromatic material is used to replenish the chromatin of the reticulum, or whether it is all unrequired and eliminated, I am unable to say. No definite centrosomic granule was noticed within the nucleus itself.

Note to Part III.

Since this part was written my attention has been called to an important paper by Debaisieux (**6a**), on the Coccidia of Lithobius. I am only able here to indicate briefly the conclusions arrived at by this author, in so far as they bear upon the chief points which have been considered in the above section. Debaisieux also finds, as do Schellack and Reichenow, that phases of more than one parasite have been confused in previous descriptions of Adelea ovata. No reference whatever is made, however, to Schellack and Reichenow's note—an omission which is to be regretted. Debaisieux agrees that there is no double (or sexual) schizogony in the true Adelea ovata; but whereas Schellack and Reichenow refer those phases which do not belong to Adelea to Barrouxia alpina, Debaisieux refers them (at any rate, those observed by Jollos) to Coccidium lacazei. I am very pleased to find that Debaisieux also entirely upholds the occurrence of a true division-centre (centrosome) and of promitotic division of the karyosome, as described by Jollos (loc. cit.); though it may be mentioned that, as regards the precise modes of nuclear behaviour and division in the later stages of schizogony, he differs in certain points from that author. Further, Debaisieux takes a view upon the nature and significance of the karyosome quite similar to that which I have mentioned above; and this author also dissents from the ideas about the karyosome propounded by Hartmann and his school.

IV. THE NUCLEAR STRUCTURE OF LEUCOCYTOZOON AND HALTERIDIUM; THE SIGNIFICANCE OF THE SO-CALLED BINUCLEATE CONDITION IN THESE FORMS, AND ITS BEARING UPON THE AFFINITIES OF THE HÆMOSPORIDIA.

THE observation of the occurrence of a distinct karyosome in certain Hæmogregarines led me to study again, from this point of view, the much-discussed nuclear condition found in the gametocytes of Leucocytozoon and Halteridium. As is now well known, female individuals of both these parasites, when stained by some modification of the Romanowsky method, show besides the ordinary nucleus, which is stained red, another very definite nuclear body, which stains much more deeply than the other, and at times appears almost black; this additional chromatic element may be either close to (in contact with) or quite separate from the nucleus. In the case of Halteridium this body has, in female individuals, the form of a conspicuous grain, but in the distinctive individuals which have been regarded as neutral or "indifferent" (which, it may be incidentally remarked, seems to occur only rarely), it is even more prominent and may be almost as large as the nucleus. In the case of male individuals, however, I have not succeeded in making out anything comparable to this structure. As I have previously described and figured the appearance shown by Halteridium fringillæ, when stained by Giemsa, I need not refer further to it; I have found exactly the same appearance in Halteridium noctuæ of the little owl.

In the case of Leucocytozoon ziemanni, the celebrated Leucocytozoon of the little owl, the additional chromatic body is very large and prominent in the female gametocytes (Pl. 10, figs. 4–6), and by no stretch of imagination can it be regarded merely as a grain! Anything more like the trophonucleus and the kinetonucleus of one of the large "blue" Trypanosomes present in the same bird might be expected to appear, in a resting, intra-cellular condition, it is impossible to suggest; and I well remember that when I first saw such individuals in preparations, really well-fixed and stained,[1] I felt no more doubt that Schaudinn's view would prove to be correct than I felt about being at Rovigno. In my opinion, this remarkable resemblance was the foundation upon which Schaudinn built up his whole theory of the ontogenetic connection between the Trypanosomes and the intra-cellular parasites (Leucocytozoon and Halteridium) of the little owl. To return to L. ziemanni, in the male gametocytes the great majority here also show no chromatin body in Giemsa-stained smears besides the large, oval, diffuse nucleus, the scattered granules of which stain faintly a pale red (fig. 7). Occasionally, however, two or three small bodies or grains, which may differ slightly in size and which stain red somewhat more deeply than the nucleus, can be made out situated close together near the margin of the nucleus, forming as it were a clump almost in contact with it (fig. 9). These small structures are really only conspicuous in individuals which are if anything over-stained. Nevertheless the elements thus occasionally indicated in the nucleus of the male forms, stained by Giemsa, are found to be practically as constant in occurrence, in films stained by iron-hæmatoxylin, as is the single large body present in the female forms.

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[1] This remark is not made with any idea of self-praise; it is by no means an easy matter to obtain Leucocytozoon well fixed and stained, even according to the Romanowsky method, so as to show the nuclear structure properly, and also the different parts of the host-cell, in their true form and relation to the parasite. It is only necessary to glance at many of the figures of different species of Leucocytozoon hitherto published to realise this. Either the parasites are hopelessly distorted and flattened out (cf. Dutton, Todd and Tobey's figs. [**2**]), or the only sign of a nucleus is a space-like area in the middle of the cytoplasm (as in some of Mathis and Léger's recent figures [**3**]); some of Wenyon's figures, too, of L. neavei (**8**) are far from giving an accurate representation of the form and nuclear details. My figures in the present paper, as also those of L. fringillinarum in a previous memoir (**9**), show approximately the true nuclear appearance, as will be seen when the condition found in wet-fixed preparations stained by iron-hæmatoxylin is discussed.

The study of these gametocytes of Leucocytozoon in films stained with iron-hæmatoxylin is most instructive. Berliner, in his account of Flagellates (1), has also given figures of the intra-cellular parasites, Leucocytozoon and Halteridium, stained in this manner, with the idea of showing that they agree with the Binucleata in the possession of two nuclei (i.e. the occurrence of nuclear dimorphism); he does not, however, give any description of the details of nuclear structure. As regards the female forms, the figures given by Berliner show, on the whole, the same appearance as that which I have found.

Taking a general view, as it were, first, of the nuclear structure of the female gametocytes (figs. 11–17), this is seen to be, in many respects, of a similar type to that of the young schizonts of "Adelea." For the most part the nucleus consists of a fairly large, central mass, which appears finely granular and stains to a moderate degree; surrounding this the same clear, almost colourless zone can usually be made out, which is present in "Adelea" (cf. figs. 1–3). Berliner figures well-marked rays traversing this narrow zone; now and again I am inclined to think I have caught a hint of the presence of one or two of these rays, but in my preparations they are so faint and elusive that it is difficult to be certain. Standing out conspicuously by reason of the intensity with which it stains is the large chromatic body, which is so prominent in Giemsa-stained smears; this is always spherical and generally surrounded by a distinct halo, as is the karyosome in "Adelea." It is usually in close association with the nucleus proper, though it may be distinctly separate from the latter, as in fig. 11, but I have never seen it so far removed as I have found it in Giemsa-stained preparations (cf. figs. 6 and 8). In two or three cases I have observed two such bodies, of unequal size, and neither so large as when there is only one, lying at opposite sides of the central mass (figs. 12, 17); the resemblance of the nuclear condition in such cases to that seen in figs. 1 and 2 of "Adelea" and figs. 24, 26, Pl. 9, of

Karyolysus, in a preceding note, is striking. This occurrence is apparently infrequent,[1] but the observation of it has considerably helped to influence me in my decision to relinquish, as no longer tenable, the view I have formerly held respecting the origin and significance of this much discussed element. Regarding this body (or bodies) in the light of the nuclear constitution existing in certain phases of "Adelea," and especially bearing in mind the fact that I have myself made known above a similar karyosomatic condition in a blood-parasite, Karyolysus, the conclusion seems to be forced upon one that here also we have to do with a true karyosomatic element, and not, after all, with a body comparable to the kinetonucleus of a binucleate Flagellate.

In regard to the finer cytological points, the nucleus of these female gametocytes differs slightly from that of the early schizonts of "Adelea" and Karyolysus, as might indeed be expected when the different nature and subsequent development of the two types of indidual is borne in mind. In those cases where there are two unequal-sized karyosomatic bodies (as I intend to designate these intensely staining elements in future), I cannot say whether they arise by the division of a single original one, in a primitotic manner, though I think this quite likely. I have not observed a spindle connecting them, but that may be because I have only found very few individuals in which there are two of these bodies. On the other hand, there is certainly a division-centre in connection with the central part of the nucleus, for not infrequently a distinct spindle (centrodesmose) is seen stretching between two granules, one of which stands out particularly from the more faintly stained chromatic material (figs. 11, 14). One of Berliner's figures also show this centrodesmose. The two granules connected by this spindle appear to be situated at the periphery of the central mass, and one is usually larger and more prominent than the other. In one instance I have observed a spindle running from the larger granule to the karyosome (fig. 16). The larger granule may be present without there being any spindle or smaller granule (fig. 13). Very frequently close to this large granule is another one of about the same size and appearance; but this latter appears to lie always outside the central mass of the nucleus, at the outer edge of the clear, surrounding zone (figs. 14–16).

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[1] It is somewhat remarkable that, in Giemsa-stained smears, I have never noticed two of these structures associated with the nucleus.

Turning now to the male gametocytes, there is always a large, oval nuclear area. As in Giemsa-stained smears, this is more usually very faintly stained (figs. 18–22)—remarkably so for a nucleus after iron-hæmatoxylin. It consists apparently of a loose reticulum with fine granules scattered throughout it. Here, also, this oval area is surrounded by a more or less distinct clear zone, but I have never, in this case, been able to make out any traces of rays crossing it, though Berliner (loc. cit.) just indicates a few in one of his figures purporting to be of male gametocytes. Berliner's figures of male individuals, however, are much less satisfactory than those which he gives of female ones; and, in fact, I am very much inclined to doubt their representing male forms at all, for reasons which I will mention shortly. In the majority of cases the outer limit of the nucleus, external to the narrow, clear zone, is more or less strongly impregnated with chromatin, in the form of distinct granules, which stain deeply, and in optical section constitute a prominent chromatic ring, sharply delimiting the periphery of the nucleus (figs. 20–23). It is noteworthy that this well-marked peripheral zone of chromatic granules in the male nucleus is apparently never to be observed in Giemsa-stained smears; it is not obvious in any of my preparations (for instance the individual of fig. 7 is on a smear made at the same time as the cover-slip preparation on which is the parasite of fig. 21), nor is it shown in any figures hitherto published. However, this zone is not always apparent, even in iron-hæmatoxylin preparations; thus the individuals of figs. 18, 19 do not show it. Although, of course, the intensity of staining and the degree of extraction have a marked effect upon the prominence of this chromatic zone and the apparent size of the granules composing it, as equally upon the appearance of the host-cell nucleus (cf. figs. 22, 23), nevertheless I do not think the seeming absence of the zone in the instances mentioned is due, to any great extent, to the technique, i.e. to a less intense staining or to an excessive amount of extraction; for one thing, both the host-cell nucleus may be more intensely stained, and the host-cell itself, i.e. its spindle-like prolongations, more readily discernible, in cases where the nucleus shows no chromatic zone than in cases where it does (cf. figs. 18, 19, and 20, 22). Again, while all the preparations made from one infected owl may show the chromatic ring prominently, in those made from another bird this feature will be either not nearly so strongly marked, or else not discernible at all; this fact also points to a difference in this condition, in different cases or at different periods. I may emphasize the fact that I have never observed it in female gametocytes.

Almost constantly associated with the male nucleus is a group of small, spherical, deeply staining elements. Very generally these are three in number; a larger, more external one and two smaller ones, of approximately equal size. The larger body is situated at the edge of the nucleus, or just outside the border or periphery (figs. 18, 19), and is often surrounded by a distinct halo. Both in position and appearance this element agrees closely with the large, conspicuous body associated with the nucleus in the female gametocytes, the only apparent difference being that it is never so large; and I do not hesitate to suggest that it represents the same organella in the male forms, namely a karyosome. Why this chromatic element should stain so much more easily and intensely with Giemsa in the case of the female individuals than it does in the male forms is another instance of the peculiar and misleading vagaries of this stain. The two smaller elements I have mentioned, which apparently represent a pair, are situated at about the limit of the central diffuse area of the nucleus, i.e. just internal to the narrow, clear zone (figs. 18, 19); (of course the disposition of these various organellæ can only be correctly ascertained when they happen to lie in the plane of optical section). The two granules are sometimes connected by a short but distinct spindle (fig. 22); and in one case (fig. 21) I have observed a spindle joining one of these granules to the larger body (karyosome).

It remains now to compare these granules occurring in the male nucleus with those described above in the female nucleus. It is highly probable that the pair of granules in the male form corresponds to the two approximately equal-sized granules seen in the female gametocytes of figs. 14–16, near the periphery of the nucleus. There is a marked agreement, moreover, between the nuclear condition shown in figs. 21 and 16, of male and female individuals respectively, where the large karyosome is still connected by a fibril with one of the two granules. A distinguishing feature in all the cases I have observed is that in the female nucleus the paired granules are radially arranged, while in the male they are tangentially arranged. The condition seen in the female individual of fig. 11, where the inner of the two granules has undergone a further unequal division, a still smaller granule remaining connected with it by a distinct centrodesmose, apparently represents a later phase which I have not seen in a male gametocyte. An important question is: Are these paired granules to be regarded as constituting kinetic elements (centrosomes) solely, or as representing small karyosomatic elements (i.e. containing also chromatin)? That they contain a division-centre does not require to be emphasised, as this fact is clear from the various centrodesmoses I have described and figured in connection with them, in both male and female nuclei. In my opinion it may be regarded as certain that the very small peripheral granule seen, for instance, in fig. 11 is a centrosome (or centriole), still in connection with its fellow one; as, however, the body at the other end of the fibril is slightly larger, it may be, perhaps, that this latter element is really a very small daughter-karyosome, possessing a certain amount of chromatin which encloses the true centriole (cf. the very small karyosome and centriole in Hepatozoon canis, see p. 203). If this be so, the other granule of the pair must also be interpreted as a small karyosomatic element, and, of course, also the corresponding pair of granules in the male nucleus.

To understand the exact significance of the somewhat complex system of divisions and resulting elements which I have described, a study of their behaviour during the further development, i. e. gamete-formation and fertilisation, would be necessary. From a consideration of figs. 14–16 it may perhaps be suggested that the more external of the paired granules, situated usually just outside the clear nuclear zone, represents a further elimination of unrequired nuclear material, possibly a kind of maturation-process; but I have no indication whether the same explanation holds good in the case of the male forms. Lastly, with regard to the large karyosome itself. Does this body contribute any of its store of chromatin to the general chromatic material during the growth of the gametocyte, or is it entirely eliminated as unnecessary? In this connection one point which I have noticed may be mentioned. The karyosome is slightly but distinctly larger in a male nucleus which does not show the chromatic zone than in one which possesses this feature (cf. figs. 18, 19, and 20, 22). This may possibly indicate, in the latter case, some augmentation or replenishment of the chromatin of the general nuclear substance and a corresponding diminution of the amount held by the karyosome.

It will be clear, I think, that in regard to the essential features the nuclear constitution of both male and female gametocytes of Leucocytozoon ziemanni shows a close agreement, and this notwithstanding the apparently pronounced differences shown when they are respectively stained by Giemsa. It is remarkable how constant in appearance, on the whole, the nuclear condition is found to be; and this fact adds, of course, to the difficulty of interpreting the elements observed While, however, the male and female nuclei of Leucocytozoon are fundamentally similar in type, there is no possibility of mistaking the one for the other, even in films stained by iron-hæmatoxylin, on account of the constant differences in detail. As I have already mentioned, Berliner's figs. 50 and 53, Pl. 29, which he regards as representing male gametocytes, do not agree at all with the characteristic appearance I have found and above described. The nucleus itself is figured as round, instead of being, as it almost invariably is, a pronounced oval in shape; and although it is somewhat larger than that of the female individuals which Berliner figures, it is nothing like the size which the male nucleus usually is. Moreover, the central area is stained more deeply, like that of the female forms, instead of being pale, even paler than the surrounding cytoplasm, as in the male forms; and lastly, there is no sign of the peripheral chromatic zone. The associated, intensely staining body is also very large, like the karyosome of the female gametocytes, and there is no indication of the small paired elements close to it. In short, I feel almost certain that the individuals figured by Berliner as of male sex are really also female forms (cf. his fig. 50 and my fig. 13, for instance).

I have dealt first with the nuclear structure of Leucocytozoon for two reasons: firstly, because in spite of its somewhat complex character it is not nearly so difficult to make out satisfactorily, on account of the large size of the parasites and the absence of pigment-grains, as is that of Halteridium, when fixed by a wet method and stained with iron-hæmatoxylin; and secondly, because it is more readily comparable with the nuclear condition found in the young forms of "Adelea" and Karyolysus. I have now to consider the nucleus of Halteridium, and will again begin with the female gametocytes. Berliner (loc. cit.) in the explanation of his figures of this parasite says nothing at all about the sex; so far as his figs. 58–60, of fairly large or adult individuals, are concerned, these certainly represent female forms. No male forms are figured, just as I maintain is the case with his figures of Leucocytozoon. The appearance of the female gametocytes, according to Berliner, also agrees on the whole with the condition I have found. In most individuals the nucleus has a close resemblance to the characteristic flagellate type of nucleus. It appears as a very clear, round area, of relatively small size, which is sharply marked off from the surrounding cytoplasm and is most probably limited by a definite membrane; in the centre is a prominent, intensely staining karyosome (figs. 24–27). Berliner figures distinct rays passing from this central karyosome to the periphery of the nucleus. I certainly believe in the presence of these rays, serving, as it were, to sling the karyosome in position, but I cannot figure them for the simple reason that, even under the best optical conditions at my disposal, I am unable to actually see them myself; and I may say that others, who have kindly scrutinised several individuals on my preparations with this object, have also failed to discern them. Nevertheless I remember perfectly well once showing one of these preparations to my colleague Miss Robertson, then working in this laboratory, and she distinctly saw some rays in two or three cases, and sketched them for me. Hence, in the determination of these extremely delicate and difficult points one's own powers of vision are an important factor. Very frequently, at one side of the nucleus and usually close to, almost in contact with the membrane is a distinct granule, which is small and does not stain black so intensely (figs. 24–27). Now and again an obvious fibril or spindle connects this granule to the karyosome in the nucleus (cf. also Berliner's figures).

This was the nuclear constitution of Halteridium as I knew it when I wrote the postscript (à propos of Berliner's figures) to the paper by Minchin and myself (**5**) on the comparison of the nuclear structure of Hæmogregarina triglæ and Trypanosoma raiæ, and when I wrote the note on Halteridium fringillæ in my first study on Avian Hæmoprotozoa (**9**). It will be generally admitted, I think, that in view of the pronounced difference shown between this type of nucleus and that of Hæmogregarines (as the latter was then known), when both were stained by a reliable cytological method, I was at the time quite justified in regarding the nuclear condition in Halteridium as corresponding closely to the karyosomatic type of nucleus seen, for instance, in a Trypanosome; and, further, in considering the definite, small associated element to represent a kinetonucleus in a "rückgebildet" condition as Berliner suggested. As a matter of fact, even until quite recently, and since I have realised the essential Coccidian nature of the nucleus of Leucocytozoon, I have been at a loss to explain this apparent resemblance of the Halteridium-nucleus to the binucleate condition and its difference from that of Leucocytozoon.

It is only within the last few weeks that I have learnt the true explanation of the matter and at last definitely settled, as I consider, the meaning of the nuclear appearance seen in Halteridium. The mistake has really been, I believe, in comparing the small associated granule, seen in films stained by iron-hæmatoxylin, with the conspicuous, deeply staining organella seen in Giemsa-stained smears, at any rate so far as regards the adult parasites. It so happens that some of my best iron-hæmatoxylin preparations of H. noctuæ are from an owl which had a heavy infection, and in which the great majority of the parasites were young, or intermediate-sized forms, relatively few being full-grown individuals. Looking over these at the time they were made, and again before writing the postscript above alluded to, I remember noting the general uniformity which was apparently presented by the nuclear structure. The small forms, the intermediate-sized ones and the few large parasites I came across all showed the karyosomatic type of nucleus, with or without the small accessory granule (and this is to be regarded, of course, as the regular condition, cf. figs. 24–27). As I then remarked, what I observed corresponded closely with what Berliner had figured. This being so, I did not undertake any systematic searching of these preparations at that time, as I wanted to continue first my study of the Avian Trypanosomes. I naturally concluded that both Berliner and I myself had seen the same nuclear condition as that which I had considered to represent nuclear dimorphism when found on Giemsa-stained smears. I remember putting aside these wet preparations of Halteridium until a convenient opportunity for their detailed study should come along, with the thought that there was at least one point which was extremely difficult to determine from an iron-hæmatoxylin preparation, namely whether a particular individual was of male or female sex; it appeared to me as if, notwithstanding the well-marked distinction between the male and female nucleus after staining with Giemsa, the nucleus of both kinds of gametocyte was really of essentially the same form and structure, and the same view seemed to have been taken by Berliner, since he did not distinguish the sex.

Having found, however, since I began to study the cytology of Leucocytozoon ziemanni, that there is a constant difference between the nucleus of the male and female gametocytes respectively when stained by iron-hæmatoxylin just as in the case when stained with Giemsa, it was necessary to return to the Halteridium and try and settle the question as regards that form. Fortunately, I have recently obtained another chaffinch with a fairly good infection of H. fringillæ, in which most of the parasites are approximating to the adult condition and whose sex can therefore be readily distinguished. This time I at once made some iron-hæmatoxylin preparations, the examination of which happily enlightened me upon the whole question, in quite as great a measure as the study of Giemsa-stained ones helped to lead me astray in the first place. With the knowledge thus gained, I turned once more to my preparations of H. noctuæ, and have now been able to ascertain that the nuclear structure here also shows the same constant differences in the male and female forms.

In figs. 28, 29 are seen male gametocytes of H. fringillæ, and in figs. 30, 31 the corresponding forms of H. noctuæ. Both the red blood-corpuscles and the adult individuals of the species of Halteridium infecting them are distinctly larger, it will be noticed, in the case of the little owl than in the case of the chaffinch. Hence the cytological details can be made out with somewhat less difficulty in the gametocytes of H. noctuæ, though of course not nearly so readily as in Leucocytozoon. It happened very fortunately that in one of my infected owls, the Halteridial parasites possessed, for some reason or other, very little pigment; many of my figures are drawn from this series of preparations, because in such a case there is no possibility of confusing the nuclear elements with pigment grains.[1] As is apparent from the figures, the nuclear structure agrees closely with that of L. ziemanni, and therefore a detailed description is unnecessary. As regards the large, oval, pale nuclear area in the male forms, I have never observed any indication of the peripheral zone of deeply staining chromatic grains, which are often so prominent in L. ziemanni; whether this is because they are not developed in the male nucleus of Halteridium, or merely because I have not succeeded in getting them to stain, I cannot say. There is, however, the same small, spherical, peripherally situated karyosomatic body, which now and again can be distinctly seen to be surrounded by a clear halo (fig. 30); and, close to it, the same dumb-bell shaped or else double centrosomic element.[2]

Turning now to the female gametocytes, it was the observation of the large, adult parasite (H. fringillæ) drawn in fig. 32 which suggested to me the explanation of the difference generally to be seen between the female nucleus of Halteridium and that of Leucocytozoon. In the individual figured, the conspicuous karyosome no longer occupies a more or less central position within the nucleus, but has passed distinctly to the outside, and bears apparently the same relation to the general nuclear substance as does the karyosome of the female individuals of L. ziemanni drawn in figs. 13–16. The chief points of difference to be noted are that the nuclear substance is here so faintly stained that it appears more like a spherical space than a nucleus; and secondly that I cannot (in this particular instance) make out any centrosomic granule. A similar condition is seen in figs. 33–35 of H. noctuæ, but two of these parasites show a distinct centrosome which is apparently intra-nuclear, though it may, of course, be lying near the upper or lower surface. The nuclear condition in this case agrees very closely with that of the female gametocyte of L. ziemanni drawn in fig. 13. I have not observed a single instance, however, where there are two granules in connection with the female nucleus of Halteridium, such as I have described as of frequent occurrence in Leucocytozoon. It is most probable, I think, that the centrosome[1] seen in figs. 33, 34 is the same element as that situated at the limit of the nucleus in figs. 24–26, but I have not found it connected by a fibril to the karyosome, where the latter has passed to the outside of the nucleus; the fibril perhaps disappears when the karyosome changes its position.

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[1] It is perhaps scarcely necessary to say that this rather unusual feature does not imply that the nuclear details themselves differ at all from the condition found in other cases, where the parasites have the usual supply of pigment grains; the nuclear structure is obviously quite similar in my figures of H. fringillæ, which show numerous grains.

[2] In the case of Halteridium, these granules are so minute that it is difficult to believe they can be anything but the actual centrioles themselves.

There can be no doubt, I think, that the smaller, intensely staining nuclear body in H. fringillæ (as seen when the parasites are stained by Giemsa), which I originally regarded as representing a kinetonuclear element, corresponds, not to the small peripheral centrosomic body seen in iron-hæmatoxylin preparations, when the nucleus has the condition shown in figs. 24–26, but to the karyosome, when this has passed to the limit of, or outside the nucleus (as in figs. 33–35); at all events so far as large or adult individuals are concerned. That this is really the case is borne out by a fact which I noticed several times, namely, that only a certain proportion of the larger female forms of Halteridium (more, I should say, in H. fringillæ, fewer in H. noctuæ) show this characteristic additional element, in Giemsa-stained preparations; whereas practically all the female individuals of Leucocytozoon ziemanni exhibit it. We arrive, therefore, at the important result that when the female nucleus of Leucocytozoon is compared with that of Halteridium in the same phase, the two are found to be of essentially the same type of structure. Their apparent dissimilarity, as frequently observed, is due to the fact that in Halteridium the karyosome retains its central position within the nucleus throughout the period of growth of the gametocyte, and does not pass to the outside until the parasite is full-grown. On the other hand, in Leucocytozoon the karyosome appears to be always at the edge of, or else outside the nucleus, even in young or intermediate-sized individuals; I have never seen it within the central nuclear mass. This expulsion of the karyosome, which doubtless represents here, as in other cases, an elimination of unrequired chromatic material or "nuclear purification," thus takes place very early in the development of the macrogametocyte of Leucocytozoon, but only at a comparatively late stage in that of Halteridium.

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[1] In the case of the female nucleus also, I think it is preferable to regard this single granule as a centrosome only. I have not observed any secondary divisions or any further elimination (?) of small karyosomatic portions, as in the female nucleus of Leucocytozoon.

The facts I have observed and described above finally settle, in my opinion, the question of the connection of Halteridium noctuæ (and equally, of course, of Leucocytozoon ziemanni) with Trypanosoma noctuæ. It appears to me that these parasites have no direct connection whatever, either ontogenetic or phylogenetic. As readers of my first study on avian parasites (loc. cit.) will be aware, I felt then compelled to relinquish the view that Halteridium and Trypanosoma were phases of one life-cycle, though I still considered that Halteridium was to be derived from a Trypanosome-like parasite, which had become permanently intra-cellular, in view of its possession (as was then thought) of the binucleate condition and of a typical Flagellate, karyosomatic type of nucleus. There may be some among those who uphold the locomotor or kinetic view of the karyosome who will even yet be inclined to say, Why should not the conspicuous, deeply staining body associated with the nucleus in Leucocytozoon and Halteridium still be regarded as representing a kinetonuclear element, perhaps in a "reduced" or non-functional condition?

The following are very strong reasons, I consider, against maintaining any longer the view that these parasites do exemplify the binucleate condition, as it is found, for example, in the case of a Trypanosome. In the first place, as I have shown in the preceding section (Note III of this series), the typical karyosome cannot be considered as a "locomotor component" at all; there is no evidence whatever that the karyosome itself stands in any special relation to the kinetic activities. Secondly, from the comparison of the true nuclear condition occurring in Leucocytozoon and Halteridium with that obtaining in the Hæmegregarine, Karyolysus lacertæ, and in certain phases of different Coccidia, it seems evident that the so-called kinetonuclear element in the first-named forms represents in reality the karyosome of these other parasites. Lastly, but by no means of least importance, when Halteridium and Leucocytozoon apparently show nuclear dimorphism, according to Giemsa-stained preparations, the nucleus itself is seen in films stained by iron-hæmatoxylin to be no longer of the well-known karyosomatic type, i.e. not comparable to the trophonucleus of a binucleate Flagellate; in short, as is clear from the study of my figures of Halteridium, the prominent extra-nuclear body is the karyosome of the nucleus.

The association of Halteridium and Leucocytozoon (and also, in all probability, of Proteosoma and the malarial parasites) along with the Hæmoflagellates in the group Binucleata has therefore to be given up. These Hæmosporidia, equally with the Hæmogregarines, must be regarded as closely allied to the Coccidia; it seems to me now that there is no longer any reason for supposing that they are derived from a binucleate form, such as a Hæmoflagellate. It has been a great disappointment to me to find that the view so elaborately worked out by Schaudinn and apparently so firmly based on facts, which I in common with many other Protozoologists adopted enthusiastically, has had to be abandoned, step by step, until the entire edifice is seen to be without any true foundation whatever. From my own work I feel persuaded that the principal if not the only basis upon which Schaudinn built was that which I have above indicated, namely the remarkable resemblance between the nuclear condition of the female gametocytes of Halteridium and Leucocytozoon, when stained by the Romanowsky method, to that which a Trypanosome might be expected to show if in a resting phase. I greatly doubt, indeed, whether Schaudinn ever saw the nuclei of these gametocytes stained by iron-hæmatoxylin; certainly no figures of individuals so stained are given in the recent published collection of his works (**7**). From the study by Minchin and myself (**4**) of this question, more especially from the standpoint of the Trypanosomes, and also from the present study of the cytology of the intra-cellular parasites, it must be admitted that no real evidence of any kind can be found to support Schaudinn's view.

ADDENDUM.

In view of the publication quite recently of a paper by Prowazek (**5A**), on the "Geschlechtsdimorphismus der Trypanosomen," I feel obliged to add a few remarks to this note. Prowazek still maintains Schaudinn's view that Leucocytozoon and Halteridium represent, in each case respectively, merely the sexual phases of a Trypanosome. He thinks that Mayer ('Arch. Protistenk.,' xxi, 1911), has sufficiently proved this idea in the case of Halteridium, and he himself endeavours to show that an actual connection exists in the case of the Leucocytozoan and Trypanosome parasites of fowls (Sumatra). So far as regards Mayer's account of the development of Trypanosomes from Halteridia, I shall have to criticise this in a later memoir; here I must confine myself to a brief consideration of the above-mentioned paper by Prowazek.

In the first place, it is impossible not to comment upon the appearance presented by the parasites in the figures on Prowazek's plates. I have pointed out above how frequently the figures hitherto given of Leucocytozoon have represented poorly fixed or stained specimens; but I do not recollect ever having seen any which are quite as bad as some of those on the plates in question. Speaking for myself, it is no exaggeration to say that, from many of the figures, taken by themselves, it is impossible to tell what they are meant to represent, so dreadfully are the parasites distorted and disorganised. It is obvious that no conclusion or interpretation can be accepted which is based upon preparations such as those from which these figures are taken.

Minchin and Woodcock (**4**), in their paper published only a month or two before Prowazek's appeared, and which presumably that author had not seen, have fully discussed the subject of the possible connection of Leucocytozoon ziemanni and the trypanosome of the little owl—the very parasites, i. e., on which Schaudinn worked—but it is not out of place to repeat here the main conclusions at which we arrived. In spite of numerous and prolonged living examinations we never observed the least sign of the passage from one form into the other—in either direction; nor is there the slightest evidence to this effect in any of our permanent preparations. The better fixed and stained these are, the more closely the Leucocytozoon agrees in form with the appearance presented in the living condition; it is remarkably uniform, and scarcely varies at all.

I may mention here a point which I have not referred to in the preceding pages of this note with regard to the true nature of the characteristic spindle-like prolongations invariably found in connection with the fully grown forms of L. ziemanni (and some other species). While I still held the view that this parasite (as also Halteridium) was a Binucleate and phylogenetically derivable from a Trypanosome-like form (**9**), I thought it most probable that, at all events in the proximal portion of these prolongations, there was some ectoplasmic layer belonging to the parasite which helped to produce the prolongations. In the case of species where the infected leucocytic host-cell remains rounded and does not develop any horn-like prolongations (as in L. fringillinarum), I regarded the ectoplasmic layer as having been completely lost. However, in my cytological study of L. ziemanni, the results of which have been given above, I have found nothing to support the presence of any ectoplasmic layer. In properly stained individuals (whether stained by Giemsa or by iron-hæmatoxylin) there is no real distinction or differentiation to be made out between the most proximal region of these prolongations, i. e. nearest to the more deeply staining cytoplasm of the parasite, and the distal portions towards the tip. In the great majority of cases the staining (which is always pale) is quite uniform in tint, only becoming gradually fainter as the prolongation narrows to its extremity (figs. 5–7, 10–12, 18, 22). Very occasionally, in Giemsa-stained smears, a space-like area can be seen, which is probably more or less artificial. In short, these prolongations undoubtedly represent solely the altered and extended cytoplasm of the infected leucocyte, this characteristic change being caused by the stimulus of the invading parasite as it grows. I gather that Wenyon (**8**) was inclined to this view in his account of L. neavei. An additional reason in favour of it is supplied by the facts in regard to the nuclear structure which I have made known, which indicate the essentially Coccidian nature of Leucocytozoon, since the Coccidia lack, of course, any differentiated ectoplasmic layer.

To refer now to certain other points raised by Prowazek, this worker considers that he obtained evidence which pointed to a Trypanosome enveloping a red blood-cell and proceeding to "take up" its nucleus. As we emphasized in our paper, we searched in vain, time after time, for signs of such a metamorphosis; the utmost we found to occur was the attachment merely of a Trypanosome to an erythroblast or a uninuclear leucocyte by one extremity, which might be either the flagellar or the aflagellar one. Two features very difficult to explain on the assumption that a Trypanosome thus passes into or becomes a Leucocytozoon are (A) the fact that the latter parasite, in its well-known form, shows always male and female individuals, whereas a similar distinction cannot be made out in the case of the Trypanosomes; and (B) the fact that quite small intra-cellular Leucocytozoan parasites occur, which certainly grow up into the characteristic adults, since intermediate-sized forms can be found. With regard to the remarkable phenomenon described by Prowazek of a parasite (a so-called "agamont") becoming separated from its original host-cell, but taking a part of the nucleus and the cytoplasm of the latter with it and penetrating with these elements into a fresh host-cell, I can only say that in all my experience I have never seen anything which could in the remotest degree suggest such an occurrence. Prowazek's figs. 2–5, pl. i, are supposed to show different stages in this process; but I cannot gather anything of the sort from them. Lastly, Prowazek also considers that the gametocytes of Leucocytozoon undergo a division, usually into two, which is considered to be longitudinal and thus to indicate the Trypanosome-character of these forms. The author says that both male and female forms may so divide, but adds the very significant remark that in some cases the two resulting individuals are not of the same sex, but one is male and the other female. From Prowazek's figures 8–17, it is perfectly clear that we have really to do here with the same condition which I have described in Halteridium (**10**), and which had been previously found in Hæmocystidium. As I discussed in that note, there can be little or no doubt that it is a question of a double infection of the host-cell, i.e. by two small individuals which may be of the same or of opposite sex, which grow up in contact with each other. And I am practically certain that the same explanation holds good also in the present case of Leucocytozoon.

In conclusion, I am sorry to say that, in my opinion, Prowazek does not bring forward a particle of reliable evidence which is of any use towards rehabilitating Schaudinn's unfortunate view.

The Lister Institute;
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EXPLANATION OF PLATES 9 AND 10.

Illustrating Dr. H. M. Woodcock's "Notes on Sporozoa. II–IV."

PLATE 9.

[All the drawings are magnified 2000 times linear. All the figures, with the exception of figs. 1, 23, 41–43, relate to Karyolysus lacertæ (Danil.). Figs. 1–18 are from preparations stained with Giemsa].

Fig. 1.—Uninfected red blood-corpuscle.

Figs. 2–8.—Young parasites, with nucleus in the earlier phase, near the middle of the body.

Fig. 3.—A free individual, which has not yet penetrated a blood-corpuscle.

Figs. 9–18.—Older forms, with the nucleus in the later phase, situated (except in fig. 14) near one end of the body.

Figs. 14 and 15.—U-shaped forms.

Figs. 16–18.—Stout, bean-like individuals, resulting from the fusion of the two arms of the U. In figs. 17 and 18 the cytoplasm of the host-cell is not visible, and the host-cell nucleus forms a "cap" round one or both ends of the parasite.

[Figs. 19–43 are from wet films, stained with iron-hæmatoxylin.]

Fig. 23.—Uninfected red blood-corpuscle.

Figs. 19–22, 24–29.—Younger parasites, showing the karyosome (or else two karyosomes) closely associated with the nucleus.

Fig. 20.—Individual showing the promitotic division of the karyosome, the two halves being still connected by a spindle.

Fig. 21.—Small, free individual.

Figs. 30–40.—Older individuals, with the nucleus near one end of the body. In figs. 31, 37, a karyosome is still associated with the nucleus.

Figs. 32–36.—Parasites showing different stages in the alteration and gradual disappearance of the unused karyosomatic material.

Figs. 30, 39 and 40.—Individuals showing no sign of the karyosome or its remains.

Figs. 37 and 38.—Individuals surrounded by a distinct envelope, and in connection with which nothing whatever can be seen of the cytoplasm of the host-cell.

Figs. 41–43.—Merozoites or very young schizonts of "Adelea ovata" (Barrouxia alpina, according to Schellack and Reichenow).

Fig. 41.—A single large karyosome is present.

Fig. 42.—The karyosome has divided into two by a promitotic division, the connecting fibril being still present.

Fig. 43.—Four karyosomes of unequal size are present, resulting from further division.

PLATE 10.

[All the drawings are magnified 2000 times linear. I am indebted to Miss Rhodes for kindly drawing figs. 4 and 7. Figs. 4–10 are from Giemsa-stained smears; all the others from iron-hæmatoxylin stained films.]

Figs. 1–3.—Merozoites or very young schizonts of "Adelea ovata"

(Barrouxia alpina), showing two or more karyosomes in connection with the nucleus.

Figs. 4–6, 8.—Female gametocytes of Leucocytozoon ziemanni. (The parasite of fig. 4 is slightly flattened out.) *c.* General cytoplasm of parasite, containing its nucleus, and the associated karyosome. *h.c.* Cytoplasm of host-cell (leucocyte), prolonged into two tails or horns. *n.* Nucleus of host cell, elongated and dumb-bell shaped.

Figs. 7, 9 and 10.—Male gametocytes of L. ziemanni. Fig. 7 shows the general appearance of the nucleus, figs. 9 and 10 a much less common appearance. (The parasite of fig. 7 is slightly flattened out.) Lettering as in fig. 4.

Figs. 11–17.—Female gametocytes of L. ziemanni, showing details of nuclear structure. (To save space, in many cases only the middle portion of the parasite and of the elongated host-cell nucleus are shown.)

Figs. 18–23.—Male individuals of L. ziemanni, to show the details of nuclear structure. (In some of these figures also the spindle-like prolongations of the host-cell are omitted.)

Figs. 24–26.—Small, intermediate-sized and fairly large female individuals of Halteridium noctuæ, to show the nuclear condition as generally seen.

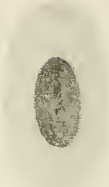
Fig. 27.—Large female form of H. fringillæ; similar nuclear condition. (Note the much smaller size of both blood-corpuscle and parasite in this case.)

Figs. 28 and 29.—Large and fairly large male gametocytes of H. fringillæ, to show the nuclear condition.

Figs. 30 and 31.—Ditto of H. noctuæ.

Fig. 32.—Large adult female individual of H. fringillæ, to show the extra-nuclear karyosome corresponding to the usual condition seen in L. ziemanni.

Figs. 33–35.—Large or fairly large female forms of H. noctuæ showing a similar or almost similar nuclear condition.



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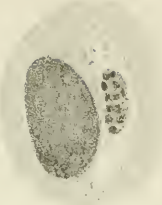

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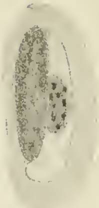

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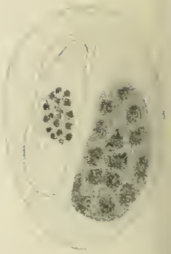

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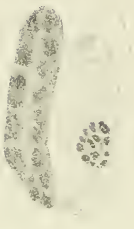

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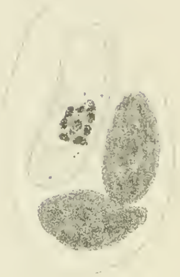

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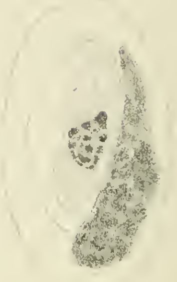

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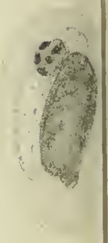

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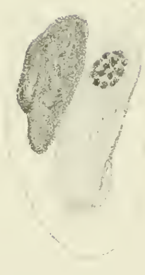

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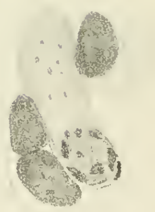

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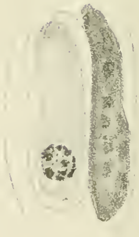

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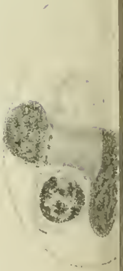

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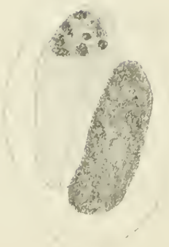

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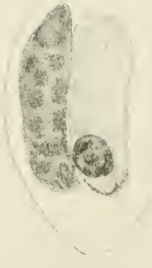

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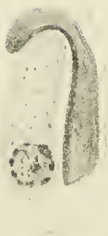

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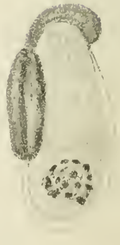

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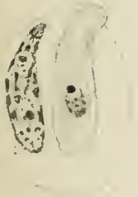
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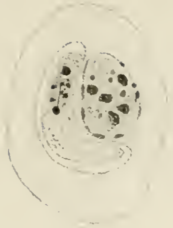
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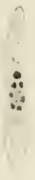
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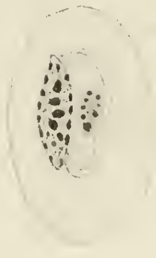
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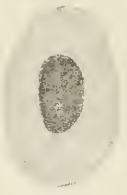
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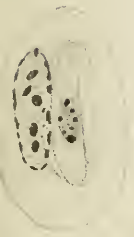
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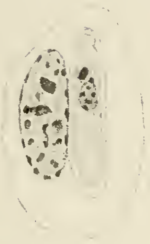
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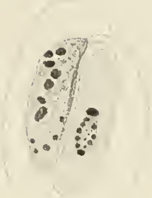
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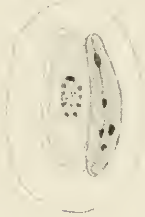
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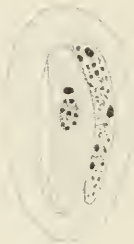
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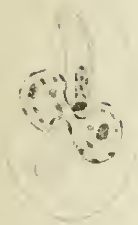
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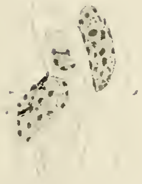
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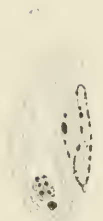
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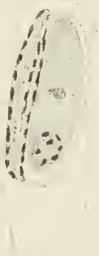
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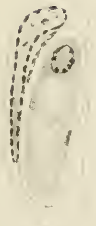
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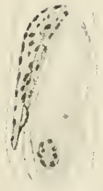
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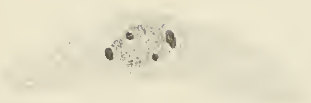
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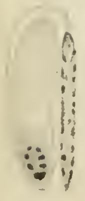
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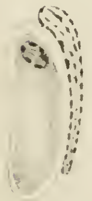
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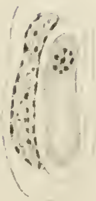
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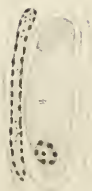
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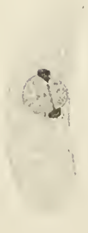
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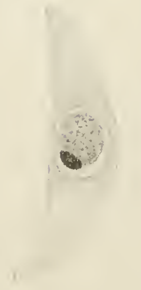
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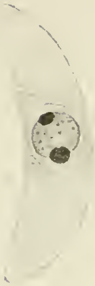

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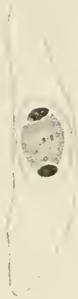

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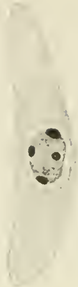

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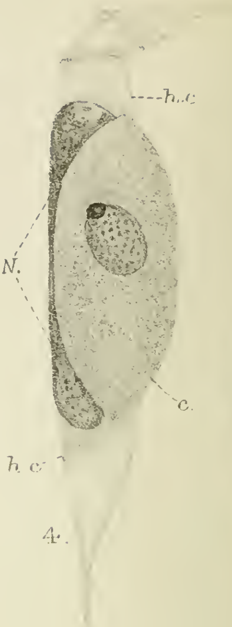

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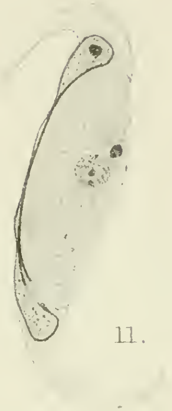

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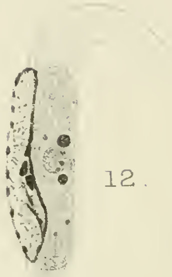

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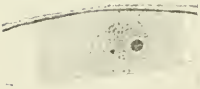

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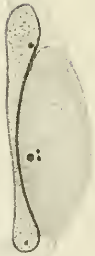

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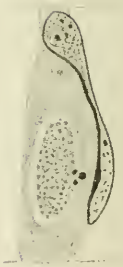

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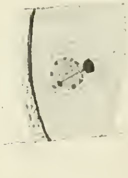

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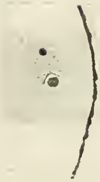

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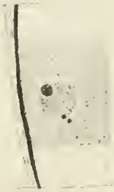

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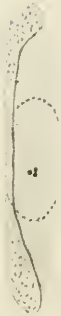

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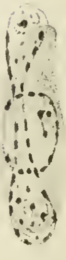

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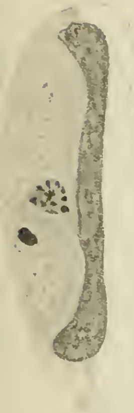

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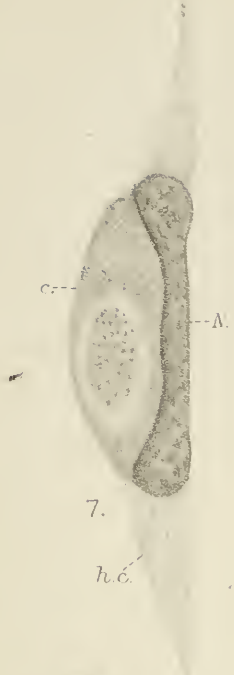

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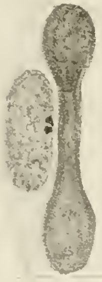

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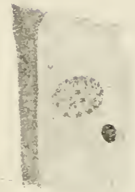

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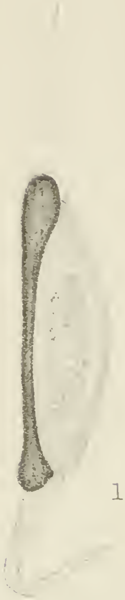

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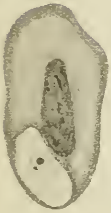

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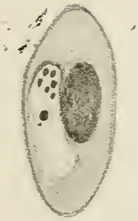

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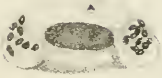

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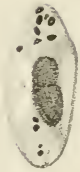

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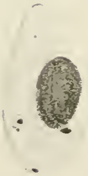

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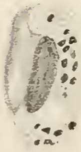

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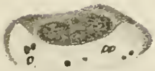

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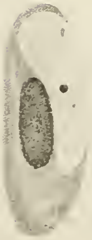

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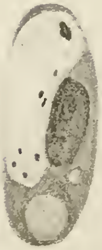

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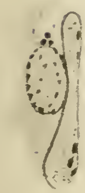

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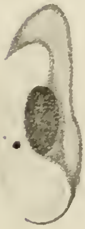

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